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ANTIPARASITE DEFENSES OF FATHEAD MINNOWS EXPOSED TO TREMATODE CERCARIAE

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ABSTRACT

Parasites exert substantial costs on their hosts. Thus, natural selection should favour behavioural defenses that reduce hosts' exposure to parasites. This prediction has rarely been tested for aquatic hosts exposed to parasites. I designed experiments to test if fathead minnows could detect cercariae of the trematode, *Ornithodiplostomum* sp. and engage in antiparasite behaviours to avoid them. Minnows exposed to cercariae formed 20.1% tighter shoals compared to water controls. Further, minnows greatly reduced their overall activity, but only when they were exposed for a second time. The latter result is important because it provides the first indication that hosts can learn to avoid parasites. Lastly, I tested if epidermal club cells play a defensive role against cercariae. Club cells did not, but other components of the epidermis, probably mucus cells, decreased cercarial infectivity by 61-68%. My results show that fish can detect, learn, and ultimately avoid aquatic larval stages of parasites.

PREFACE

"Nothing shocks me. I'm a scientist."

-Indiana Jones

ACKNOWLEDGEMENTS

greatest thanks go to my brother Garrett and my parents Bruce and Holly James. Without them, none of this is possible.

I dedicate this thesis to the many fathead minnows and snails that endured countless hours of grief and made the ultimate sacrifice for this research. The day you lack sympathy for your experimental subjects is the day you should abandon science.

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Chapter 1. General Introduction

Parasites inflict profound and pervasive costs on their hosts. Experiments involving the addition of parasites to groups of hosts under field and laboratory conditions show reductions in physiological, developmental and reproductive performance compared to uninfected controls (reviews by Scott, 1988; Combes, 2001; Poulin, 2007). Likewise, parasite removal experiments show that hosts that have been treated to remove parasites tend to outperform those with normal parasite loads (Hudson, 1998). Results from these types of experiments verify predictions of theoretical models (e.g., Anderson and May 1978; Poulin, 2007) that indicate the effects of parasites on the fitness of host individuals are significant and important.

Given these significant costs to host individuals, natural selection should favour strategies that allow hosts to reduce their rates of exposure to parasites, or to limit the magnitude of their effects. In a review, Goater and Holmes (1997) emphasized that while traits associated with the host immune system are obvious candidates for parasite-mediated natural selection, selection on other traits may be just as important. This is especially so in the face of mounting evidence for the extreme costs of immunity, from direct costs associated with the metabolic costs of mounting an immune response (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2003) to immunopathology (Wakelin, 1996). One class of strategies suggested by Goater and Holmes (1997) to address costs of infection, perhaps even independent of host immunity, is for selection to operate on traits associated with host tolerance to infection. Another class of strategies is for hosts to

avoid infection altogether by reducing rates of exposure to parasites. This strategy should be favoured when the costs of infection are high or the costs of immunity are high, and especially when the costs of both are high.

Hart (1994) was among the first to emphasize the importance of parasite-mediated selection for parasite avoidance behaviours, especially in the face of costly immunity. Combes (2001) also emphasized avoidance behaviours in the context of 'exposure filters' that may limit infection rates. Such behaviours range in complexity from simple adjustments to host movement, posture and habitat choice, for example to avoid biting arthropods, to sophisticated avoidance behaviours linked to fine-tuned parasite-detection strategies. For example, cattle can detect larval ticks and avoid grazing in areas with high densities of ticks compared to areas with low densities (Sutherst *et al*., 1986). Moreover, sheep prefer to graze in areas without faeces containing larvae of the intestinal nematode parasite, *Ostertagia circumcincta* (Cooper *et al*., 2000). Thus, there is empirical support for the notion that hosts can reduce their rates of exposure to some types of parasites.

While emphasis has been placed on the avoidance behaviours of hosts exposed to ectoparasites, fecal patches, and parasitoids, much less attention has been devoted to parasite avoidance behaviours in aquatic systems, especially those involving fish (Barber *et al*., 2000; Wisenden *et al*., In press). Initial support comes from a study by Poulin and FitzGerald (1989a) who were the first to provide empirical evidence that fish engage in parasite avoidance behaviours, in this case shoaling, and that these behaviours were beneficial to individual hosts. They showed that three-spined stickleback (*Gasterosteus*

aculeatus) and blackspot stickleback (*G. wheatlandi*) preferred to participate in larger

shoals in the presence of the branchiuran *Argulus canadensis* and that individual fish in

larger shoals were attacked less frequently. Poulin and FitzGerald (1989b) also showed

that sticklebacks avoid areas in laboratory aquaria occupied by *A. canadensis*. Poulin *et

al*. (1991) provide another empirical example of parasite avoidance behaviours in fish,

showing that the time that brook trout (*Salvelinus fontinalis*) spent active in laboratory

aquaria was positively associated with exposure to the copepod parasite *Salmincola

edwardsii*. Thus, inactive fish acquired fewer parasites. These are solid examples of

parasite avoidance behaviours of fish. However, in all cases the parasites are visible and

likely easily detectable ectoparasites.

Some empirical support also exists for the detection and avoidance of less

conspicuous larval stages of aquatic parasites. Karvonen *et al*. (2004) demonstrated that

rainbow trout actively avoid refugia containing cercariae of the trematode, *Diplostomum

spathaceum* and that the time spent under the shelter positively correlated with increased

rates of exposure. In a laboratory experiment, larval green frogs, *Rana clamitans*, and

wood frogs, *Rana sylvatica*, reduced their activity by 25-33% when exposed to cercariae

of the trematode *Echinostoma* sp. (Thiemann & Wassersug, 2000). Some hosts avoid

depositing eggs in areas where trematode cercariae or individuals infected with

metacercariae are present (Lowenberger & Rau, 1994; Kiesecker & Skelly, 2000). These

studies indicate that there is initial support for the existence of general avoidance

behaviours in fish (refuge use and general activity) and other aquatic animals, but too few

studies have been completed on too few host/parasite interactions to make

generalizations. Thus, I have little understanding of the degree to which even the most pathogenic aquatic parasites are detectable in the aquatic medium. Further, in the case that they are detectable, I do not know if the level of detectability is strong enough to produce behaviours that reduce infection rates.

Shoaling is a typical behaviour that many species of fish use to lower an individual's risk of both predation and parasitism (see review by Pitcher & Parrish, 1993). Antipredator functions of shoaling that include risk dilution, increased vigilance, and attack confusion should apply equally to parasite risk (review by Wisenden *et al.*, In press). As noted above, sticklebacks exposed to ectoparasites prefer to join larger shoals and shoaling lowers the risk of parasite attack for individuals (Poulin & FitzGerald, 1989a). Because trematode cercariae are motile and actively seek-out their fish intermediate hosts, shoaling should confer similar benefits to fish exposed to cercariae. Sweeting (1974) observed an increase in shoaling behaviour of several species of fish in the presence of trematode cercariae, *Diplostomum* sp., and predicted that individual fish within shoals should reduce their rates of exposure. However, the hypothesis that shoaling reduces an individual's risk of cercarial infection has not been experimentally tested.

Although a small number of studies have shown that aquatic hosts can detect cercariae and then elicit behavioural responses to them, the role of learning in these responses has also not been evaluated. This is an important shortcoming. Predator-naïve fish do not recognize predators as dangerous until after they have had an opportunity to

associate an olfactory (Chivers & Smith, 1994a), visual (Chivers & Smith, 1994b) or

auditory (Wisenden *et al*., 2007) stimulus with a predation event. Commonly, the

releasing stimulus for this form of learning is chemical alarm cues released from injured

epidermal tissue that occurs as a natural consequence of predatory attack (see below).

The same classes of chemical cues are reportedly released following exposure of juvenile

rainbow trout to cercariae of *Diplostomum spathaceum*, even when the odour of the

cercariae themselves invoked no response (Poulin *et al*., 1999). Thus, the sophisticated

learning mechanisms that arose to mediate risk of predation should be applied equally to

risk of infection. Presumably, fish and other aquatic hosts are continually exposed to

variable numbers of cercariae over their lifetimes. For example, fathead minnows

(*Pimephales promelas*) are exposed to hundreds of cercariae per week, of multiple

trematode species, during late summer (Sandland *et al*. 2001). Therefore, learning to

avoid cercariae would presumably be adaptive. The hypothesis that hosts learn to avoid

trematode cercariae, has also not been tested.

One possible additional strategy to reduce rates of infection of skin-penetrating

parasites (e.g., trematode cercariae, myxozoans, fungi) is at the point of contact – the

epidermis. Components within the epidermis, such as mucus, are often linked with the

immune system and have been reported to reduce infection of a variety of pathogens and

parasites (James, 1991; Shephard, 1994; Buchmann, 1999). Karl von Frisch (1941) first

discovered that the skin of certain fishes contain compounds, originally termed

'*Schreckstoff*", that cause 'fright' reactions in nearby conspecifics. Pfeiffer (1967) took

this one step further by observing that '*Schreckstoff*' originated from epidermal cells,

coined 'alarm substance' cells or club cells, found throughout the epithelium of fish from the Superorder Ostariophysi. The contents of these cells, more commonly known as alarm cue, are a source of public information that nearby fishes can use to assess environmental risk (review by Smith, 1992; Poulin *et al*., 1999). The antipredator benefits of club cells for receiver fish detecting local alarm cue is well known, yet the fitness benefits that the sender accrues are under considerable study (Chivers *et al*., 2007). Chivers *et al*. (2007) were the first to test the hypothesis that pathogens are a likely selective force responsible for the origin and maintenance of club cells (Mathis *et al*., 1995; Chivers *et al*., 1996).

Initial results from tests of the antipathogen hypothesis provide empirical support that club cells are linked to the immune system and contain antipathogenic agents (Chivers *et al*., 2007). Chivers *et al*. (2007) found that fathead minnows produce more club cells following infection of trematode cercariae (*Telorchis* sp.) and common water moulds (*Saprolegnia ferax* and *S. parasitica*) but do not respond similarly to general epidermal damage, indicating a pathogen-specific cellular response. Another important result is that homogenized ostariophysan skin extract reduces the growth rate of *S. ferax* by approximately 20% compared to water controls and a low concentration of non-ostariophysan skin extract that lacks club cells (Chivers *et al*., 2007). Furthermore, fish exposed to environmentally relevant levels of cadmium, a heavy metal that acts as an immunosuppressant in vertebrates, were unable to respond by producing club cells similar to water controls when challenged with *S. ferax* (Chivers *et al*., 2007). The authors provide compelling evidence that club cells have evolved as a defense against

pathogens. However, the hypothesis that club cells are an epidermal defense to reduce

exposure to skin-penetrating cercariae requires further testing.

Host-Parasite System

Ornithodiplostomum sp. is an unidentified diplostomatid (Digenea: Strigeidae) trematode.

Morphologically, developmentally, and ecologically, it is very similar to *O. ptychocheilus*

(Goater, unpublished observations). Thus, it utilizes pond snails (*Physa* spp.) as first

intermediate host, various cyprinid minnows as second intermediate host, and various

fish-eating birds as definitive host. In Alberta lakes and ponds, metacercariae of

Ornithodiplostomum sp. are very common in fathead minnows. Prevalence in most

populations of minnows is 100% and individual fish typically contain hundreds of

encysted larvae (Goater, unpublished observations). Metacercariae of this trematode are

sympatric with *O. ptychocheilus*. Thus, virtually all minnows infected with

Ornithodiplostomum sp. are concurrently infected with *O. ptychocheilus* (Sandland *et al*.,

2001; Goater, unpublished observations). The presence of two encysted 'forms' of

Ornithodiplostomum metacercariae has been recognized for many years, one encysting in

the optic lobes of the brain, the other encysting in the body cavity (Hoffman, 1958;

Sogandares-Bernal *et al*., 1979, Sandland *et al*., 2001). Recent experimental studies have

confirmed that the two forms are separate, congeneric species (Goater, unpublished

observations). *Ornithodiplostomum ptychocheilus* utilizes the snail *Physa integra* as first

intermediate host and the metacercariae are obligate specialists within the nervous system

of fathead minnows. *Ornithodiplostomum* sp. utilizes the snail *Physa gyrina* as first

intermediate host and its metacercariae are obligate specialists within the liver and body cavity.

The precise costs to fathead minnows infected with *Ornithodiplostomum* sp. have not been evaluated. However, preliminary data indicate that minnows infected with metacercariae of *Ornithodiplostomum* sp., incurred significant metabolic costs 17 days following exposure to 20 or 120 cercariae compared to water controls. This preliminary result parallels results from other studies, including those involving *O. ptychocheilus* (e.g., Shirakashi & Goater, 2005) and the broad negative effects of infection on fish hosts (e.g., Lemly & Esch, 1984)

A key feature related to my thesis is that *Ornithodiplostomum* sp. is amenable to experimental manipulation in the same manner as the large number of studies completed by Goater and colleagues on *O. ptychocheilus* (Sandland & Goater, 2000; Sandland & Goater, 2001; Shirakashi & Goater, 2001; Shirakashi & Goater, 2002; Schleppe & Goater, 2004; Goater *et al*., 2005; Shirakashi & Goater, 2005). I used *Ornithodiplostomum* sp. for practical reasons. First, my proposed experiments required the use of large numbers of cercariae that could reliably be made available only from experimentally-infected snails. Further, the intent was to maintain the life-cycle in the Goater laboratory, yet transport infected snails to the Wisenden laboratory in Minnesota. In terms of practicality, *P. gyrina* exposed to *Ornithodiplostomum* sp. larvae have high survival under laboratory conditions; *P. integra* exposed to *O. ptychocheilus* do not (Goater, unpublished observations). Thus, I utilized the fathead

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minnow/*Ornithodiplostomum* sp. interaction in my thesis because I could be reliably assured of the availability of the thousands of cercariae per day required for my laboratory and field experiments.

In addition to their ease of use for experimental infections under laboratory and outdoor conditions, *Ornithodiplostomum* sp. second intermediate host, fathead minnows, are also an excellent model species. Minnows are common test subjects in both fish ecology and behaviour studies, which are fields directly relevant to my thesis. First, they are a model system in the study of predator avoidance behaviours, specifically shoaling behaviour and reduced activity (Hager & Helfman, 1991; Chivers *et al*., 1995; Mathis & Smith, 1993). In the presence of their common freshwater predator, northern pike (*Esox lucius*), fathead minnows form tighter shoals and this behaviour corresponds with an increase in survival (Mathis & Smith, 1993). Second, they are the main model system in studies designed to understand the ecological and evolutionary significance of alarm cells in fish (Smith, 1992; Mathis & Smith, 1992; Wisenden *et al*., 1995; Magurran *et al*., 1996; Chivers *et al*., 2007).

Thesis objectives

In Chapter 2, I provide the first experimental test of the hypothesis that shoaling can reduce an individual fish's risk of exposure to trematode cercariae. I first compare the magnitude of the shoaling response of groups of fathead minnows exposed to *Ornithodiplostomum* sp. cercariae to those exposed to water alone. In a second

experiment, I confined groups of minnows into artificial shoals within outdoor mesocosms and then exposed them to snails releasing *Ornithodiplostomum* sp. The overall intent of these experiments is to assess whether minnows exposed to cercariae form tighter shoals, and if so, if this behaviour reduces a minnow's risk of exposure.

In Chapter 3, I test the hypothesis that aquatic hosts detect cues released from infective parasites and then engage in appropriate avoidance behaviours. Specifically, I test the ability of fathead minnows to recognize, and subsequently avoid, chemical cues of *Ornithodiplostomum* sp. cercariae. In this chapter, I am especially interested in testing whether potential behavioural responses to cercariae are innate, or require previous infection experience to associate cercariae with risk.

Chapter 4 provides one of the first experimental tests of the antiparasite hypothesis proposed by Chivers *et al*., (2007). This hypothesis makes two important predictions. First, if club cells play a role in protection of fish against skin-penetrating parasites, then fish exposed to cercariae should invest in more (or larger) club cells. To test this prediction, I compared the density of epidermal club cells in minnows exposed to *Ornithodiplostomum* sp. cercariae to those exposed to water only. The second prediction is that the contents of club cells should reduce rates of parasite transmission (Chivers *et al*., 2007). I tested this prediction by evaluating the infectivity of *Ornithodiplostomum* sp. cercariae in minnows. For this experiment, cercariae were exposed to varying concentrations of club cell containing skin extract (and a water control) and their infectivity evaluated by counting metacercariae.

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Chapter 2. Shoaling as an antiparasite response for fathead minnows exposed to trematode cercariae

ABSTRACT

For many species of fish, the costs of group-living (i.e., shoaling) can be offset by reduced risk of predation. Whether a shoaling response provides protection for individuals exposed to parasites is poorly known, especially for aquatic endoparasites that have obligate penetrative stages. Fathead minnows are group-living cyprinids that have a well-characterized shoaling response. They also serve as intermediate hosts to a variety of trematodes, the cercariae of which penetrate the epidermis. Here, I tested the shoaling response of groups of fathead minnows exposed to known numbers of cercariae of the trematode *Ornithodiplostomum* sp. in aquaria. On average, fathead minnows exposed to cercariae formed 20.1% tighter shoals compared to controls that were exposed to water alone. In a second study, I tested if group-living conferred protection for individuals exposed to cercariae in semi-natural conditions in outdoor mesocosms. Minnows were confined within cages configured into either groups of six individuals or alone and then placed into mesocosms that contained snails infected with *Ornithodiplostomum* sp. Individual minnows constrained to grouped containers had 13.3% fewer encysted metacercariae (mean intensity = 103.3, SD = 43.8) than solitary individuals (mean intensity = 119.2, SD = 62.2), but this difference was not significant. Taken together, these results show that minnows formed tighter shoals in response to cercariae, but group-living is not sufficient to reduce rates of cercariae infection.

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INTRODUCTION

Shoaling is defined as a social behaviour of fish that can be measured in terms of

cohesion amongst conspecifics (review by Pitcher & Parrish, 1993). This type of group-

living is often preferred by prey fish because it has several important functions that may

either reduce an individual's risk of predation and/or increase its foraging success

(Alexander, 1974; Bertram, 1978; Slobodchikoff, 1984; Pitcher & Parrish, 1993).

Because vigilance is proportional to group size, individuals in groups can increase the

likelihood of food location and/or predator detection while also allowing them more time

to allocate to non-vigilance activity (Pitcher *et al*., 1982; Ydenberg & Dill, 1986; Godin

et al., 1988). Shoaling can also provide a means to facilitate social learning through

information sharing between conspecifics (review by Brown, 2003). Finally, groups

statistically dilute the probability of predator attack in proportion to group size, and may

also confuse predators by making it difficult to visually isolate individual prey (Landeau

& Terborgh, 1986; Pitcher & Parrish, 1993). Given the range of advantages that

individuals can experience by living in groups, the frequency of shoaling behaviour in

fish is not surprising.

Certain antipredator functions of shoaling such as early detection, information

sharing, risk dilution and attack confusion should apply equally to parasite risk. Initial

evidence for a linkage between host group size and infection comes from a meta-analysis

showing that parasite prevalence and intensity increased as group size increased, at least

for parasites that actively seek their hosts (Cote & Poulin, 1995). In one of the few

empirical tests of this idea, Poulin and FitzGerald (1989) showed that sticklebacks

(*Gasterosteus* spp.) preferred to shoal in the presence of *Argulus canadensis*, a large

crustacean ectoparasite, and as group-size increased, shoaling lowered the numbers of

parasite attacks on individual fish. However, fish are exposed to an enormous diversity

and number of parasites other than free-swimming, visible ectoparasites. For example,

the larval stages of some trematodes, known as cercariae, are free-living and actively

seek out their intermediate hosts (review in Haas, 1994). Although anecdotal evidence

supports the idea that shoaling can reduce the exposure of gudgeon (*Gobio gobio*),

rainbow trout (*Oncorhynchus mykiss*), and roach (*Rutilus rutilus*) to trematode cercariae

(Sweeting, 1974; Karvonen *et al*., 2004), the anti-cercariae role of shoaling has not been

experimentally evaluated.

Fathead minnows (*Pimephales promelas*) are an ideal host to evaluate the linkage

between shoaling and exposure to cercariae. First, they are typical group-living

freshwater fish and are well known to form tight shoals in response to predation (Hager

& Helfman, 1991; Chivers *et al*., 1995; Mathis & Smith, 2003). Mathis & Smith (2003)

found that not only do minnows shoal in the presence of a common freshwater predator

(*Esox lucius*) but that the magnitude of the shoaling response was positively correlated

with their survival time. Further, minnows are straightforward to maintain under

laboratory and semi-natural conditions and their shoaling responses in the laboratory

parallel those evaluated under natural conditions (Krause *et al*., 2000). Lastly, two of

their most common and abundant trematodes, *Ornithodiplostomum ptychocheilus* and

Ornithodiplostomum sp., are amenable to experimental manipulation under lab conditions

(Chapter 1).

Metacercariae of *Ornithodiplostomum* sp**.** are common and abundant in lakes and ponds in Alberta, Canada that contain fathead minnows (Sandland *et al*., 2001; Goater, unpublished observations). Metacercariae encyst within the body cavity. In Alberta, this species is never found in sympatric species of fish. Young-of-the-year are exposed to cercariae of this species within their first few months of life, typically entering their first winter with 10-50 metacercariae (Sandland *et al*., 2001). Between July-October of their second year, they are typically exposed to hundreds of cercariae. Fish are exposed to cercariae that are released from infected snails, *Physa gyrina*. Fish-eating birds are the final host.

In this study, I performed two experiments to test the hypothesis that shoaling reduces an individual minnow's risk of cercariae infection. In the first experiment, I compared the magnitude of the shoaling response of minnows exposed to known numbers of cercariae of *Ornithodiplostomum* sp**.** in laboratory aquaria to the shoaling response of unexposed minnows. In a second experiment, I evaluated whether individual fish constrained into artificial shoals reduce their rates of exposure to cercariae compared to individuals constrained alone within containers.

METHODS

Shoaling response of fathead minnows exposed to cercariae

The aim of this experiment was to determine if minnows shoal in the presence of

Ornithodiplostomum sp. cercariae. This experiment was conducted at Minnesota State

University of Moorhead, MN, USA between July - August, 2006. Two hundred Lab-

reared fish (30-d-old, approximately 1.5-2 cm in length) were obtained from the U.S.

EPA National Health and Environmental Effects Research Laboratory, Duluth,

Minnesota, USA. Minnows were maintained in 2 separate 190-L tanks with a constant

12:12 L:D photoperiod for 11-d prior to the experiment.

For experimental infections involving cercariae of *Ornithodiplostomum* sp., I

followed the methods of Sandland and Goater (2000). Eight, 1-d old chickens were fed

metacercariae collected in May, 2006 from the infected viscera of wild minnows obtained

from Gold Spring Lake, southern Alberta, Canada. Trematode eggs were collected from

chick faeces 5 days post-infection and F1 pond snails (*Physa gyrina*), lab-reared from

parents collected in June of 2006, were exposed to the resulting miracidia. Snails were

housed in 2-L plastic containers at 18° C, with a 12:12 L:D photoperiod and were fed

romaine lettuce daily. To obtain cercariae, nine infected snails were isolated in small

vials with dechlorinated tap water for 2-hr under direct artificial light. Cercarial counts in

three 1-mL aliquots were averaged to estimate the volume of water needed to obtain a

dose of 400 cercariae.

Groups of 5 randomly-selected minnows were exposed to one of two types of stimuli: water containing 400, 2-hr old cercariae or to water alone. Each of the exposure treatments was replicated 15 times. The ordering of the exposure trials was determined at random and 5 trials of each treatment were completed each day over a 3-d period. The trials occurred within 5, 37-L tanks that were divided in half with black Plexiglas (Fig. 2.1). A 5 cm X 5 cm grid was drawn on the front of each tank and the back and sides were painted to prevent visual interaction between minnows in adjacent aquaria. Tanks were filled with dechlorinated tap water to the top-most grid. Each half-tank was equipped with a plastic tube (stimulus hose) that facilitated the entry of stimuli. The stimulus hose was then attached to the top of another plastic air supply hose that was affixed with an airstone. The turbulence created by the airstone masked the entry of the stimulus and the current created by the air allowed for quick dispersion of the test stimuli. The opening of the stimulus hose coupled with the air supply hose was affixed along a back corner of each aquaria.

Five minnows were placed into each half-tank 18-hr before the trials. The minnows were fed 1-hr prior to the start of each trial. Preceding each trial, 40-mL of water was removed from each tank using a syringe connected to the stimulus tube and then discarded. The purpose of this was to remove any stagnant water from within the tube. Subsequently, another 40-mL of water was removed from each tank and retained (flush water). Using a different syringe, fish were then exposed, via injection, to one of the two stimuli treatments (cercariae or water). Each stimulus was followed by an injection of 40-mL of flush water to ensure all stimuli entered the tank. The injection

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was done slowly to minimize disturbance of the test minnows. Tanks were thoroughly
washed and rinsed with dechlorinated water following all trials. Trials were video
recorded behind a black plastic blind for a total of 10.5-min. The 10.5-min period was
separated into a 285-s pre-stimulus period, a 60-s stimulus injection period, and a 285-s
post-stimulus period. Using the video records, the cohesion of the group of 5 minnows
was assessed every 15-s for both pre-stimuli and post-stimuli periods.

Risk of trematode infection in artificial shoals

The aim of this experiment was to determine if individual minnows confined within
artificial shoals with conspecifics acquire fewer *Ornithodiplostomum* sp. than fish that
were not in shoals. This study was carried out at the University of Lethbridge between
August and September, 2007. One hundred, lab-reared fathead minnows were purchased
from a biological supply house (Aquatic Research Organisms, Hampton, New
Hampshire, USA) and were housed in a 280-L tank with a constant 16:8 L:D
photoperiod. Stock minnows were fed ad libitum on commercial flake food for 6-wk
before the experiment.

Eight, 1200-L livestock watering tanks (108 cm diam, 120 cm tall) were used in
the experiment. A description of the methods used to set-up the tanks is provided in
Pearson and Goater (2008). In brief, the tanks were filled with irrigation water on 1-May.
On the same day 800-g of air-dried reeds (*Tyhpa* sp.) was placed in each pond to promote
the growth of algae and to provide a substrate for the growth of diverse aquatic flora and

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fauna. Three weeks later, 2-L samples of concentrated zooplankton collected from local ponds and lakes was added to each tank. The purpose of this procedure was to create a semi-natural aquatic environment that would promote the growth and survival of snails and also minnows.

In this experiment, exposure to *Ornithodiplostomum* sp. cercariae was facilitated by the addition of 2 snails that had been infected in the laboratory in May, 2007. The infection status of each source snail was checked on 3-August following my standard protocols and then 16 size-matched snails were selected for addition to the 8 tanks. Pairs of snails were selected at random and added to the tanks on 4-August. Minnows were added 4-d later.

Minnows were caged and assigned at random into one of two spatial configurations: "shoal" (6 minnows) and "non-shoal" (1 minnow). A single "shoal" and 6 "non-shoal" cages were assigned to each tank for a total of 8 replicates per shoal configuration. Each cage was distanced approximately 30 cm from all other cages and the position of the shoal cage amongst the non-shoal cages was determined randomly (Fig. 2.2).

The cages were constructed from screen material that my preliminary observations indicated would prevent the minnows from escaping, yet would not impede cercarial activity. Thus, cages were constructed from Trical plastic netting (3 mm mesh

size) and fastened together with monofilament fishing line. Each shoal cage confined 6

minnows and measured 4 x 8 x 12 cm (H x W x L) for a total volume of 384 cm^3. Cages

designed to hold a single minnow (4 cm X 4 cm X 4 cm) were constructed so that their

overall dimension was 1/6 the total volume of the shoaling cages. All cages were

suspended mid-column by a monofilament line anchored by a metal weight and held aloft

by a plastic float (Fig. 2.2).

Minnows were left undisturbed in the tanks for 4 days, during which they

presumably fed on the abundant phytoplankton and zooplankton that was present in the

tanks. Following this period, the minnows were transferred to separate 37-L tanks for 4-

wk and then humanely killed and necropsied to determine metacercariae intensity.

Analyses

Prior to analyses, all data on metacercariae counts and host lengths were tested for

normality using the Shapiro-Wilk test. The numbers of metacercariae in individual fish is

often positively correlated with fish body size. Thus, for all analyses, I first evaluated

whether mean fish length differed between the various treatments using a one-way

ANOVA. For the first experiment, the degree of group cohesion amongst minnows was

assessed by measuring the minimum number of grid squares, including empty squares,

connecting all 5 minnows in each aquarium. This measurement was considered the

"shoaling index" (SI) for each group of minnows and thus a smaller SI translated to

tighter group cohesion. Grid squares were considered connected by either their sides or

corners. The total change in SI, or the degree of a groups shoaling response to each

stimulus, was determined by subtracting the pre-stimulus SI from the post-stimulus SI.

The average change in SI between fish exposed to water compared to those exposed to

cercariae was compared using a one-way ANOVA.

The analyses of differences in metacercariae intensity for caged fish were less

straightforward. While metacercariae counts from individual fish in the single cages are

independent, counts from fish in the shoaling cages are not. To avoid pseudoreplication,

I determined mean intensity of metacercariae calculated from the metacercarial counts

from each surviving fish in a shoal cage. This mean was used as my metric of 'mean

metacercariae intensity in shoal cages per pond'. Because of variable cercarial-release

rates by snails between ponds, the average metacercariae intensity for shoal and non-

shoal treatments within each pond were matched and differences between shoaling

regimes were then evaluated with a paired t-test. Within each pond, metacercariae

intensity was compared between shoaling vs. non-shoaling configurations using a non-

parametric Wilcoxon signed-rank test.

RESULTS

Shoaling response of fathead minnows exposed to cercariae

There was no significant difference in minnow length between cercariae-exposed and

non-exposed treatments (one-way ANOVA, $F_{1, 50} = 0.16$, $p = 0.69$). Shoaling index data

were normally distributed (Shapiro-Wilk test, $W = 0.95$, $p = 0.22$). The overall change in

mean shoal cohesion between pre-stimuli and post-stimuli periods was significantly higher for fish exposed to cercariae than for controls (one-way ANOVA, $F_{1, 28} = 9.20$, $p = 0.0052$, Fig. 2.3). Overall, minnows exposed to cercariae in the aquaria formed approximately 20.1% tighter shoals than minnows exposed to water alone.

Risk of trematode infection in artificial shoals

Of the 96 fish used in this experiment, 77 (80%) survived the 4-wk period of metacercariae development prior to dissection. Minnows that did not survive the development period were not included in the analyses. Mean minnow length was not significantly different between the two shoal configurations ($F_{1, 14} = 0.64$, $p = 0.44$).

Mean metacercariae intensity varied by approximately 3X between individual ponds (Table 2.1). Three ponds contained fish with mean intensities greater than 100 metacercariae per host. The remaining ponds tended to contain fish with fewer than 80 metacercariae in the body cavity. Mean metacercariae intensity within ponds was not correlated with the magnitude of variation in metacercariae counts ($r = 0.14$, $p = 0.21$) (Table 2.1). Likewise, the frequency distribution of metacercariae counts in the total sample of 39 shoal fish and 38 non-shoal fish showed that individuals were exposed to 20-356 cercariae over the course of the 4-d exposure period (Fig. 2.4). When ponds were treated as independent replicates with pooled averages, mean parasite intensity did not differ between shoal and non-shoal configurations (paired t-test, $df = 7$, $t = 0.97$, $p = 0.36$, Fig. 2.5). When the assumption of non-independence in artificial shoals was relaxed,

mean metacercariae intensity did not overcome the threshold of significance between

shoaled and non-shoaled fish (one-way ANOVA, $F_{1,75} = 0.52$, $p = 0.47$). The ability to

detect an effect of shoaling with this experiment was quite low, probably due to the

unforeseen high levels of variation between and within ponds ($\sigma = 53.8$, $\delta = 20$, power =

0.28).

DISCUSSION

Results from the laboratory experiment supported the shoaling hypothesis. Thus,

minnows formed tighter shoals when exposed to cercariae of *Ornithodiplostomum* sp.

This result parallels the findings of similar behaviour observed in shoaling sticklebacks

exposed to ectoparasites (Poulin & FitzGerald, 1989). This is an important result because

it indicates that minnows can detect the presence of cercariae in the water column, and

take evasive action that might lead to reduction in exposure.

The ability of hosts to detect invading parasites is an important pre-requisite for

behavioural avoidance (Wisenden *et al.*, in press). There are a number of possible

mechanisms that minnows could use to detect cercariae of *Ornithodiplostomum* sp. that

could ultimately lead to a defensive response. Although direct visual assessment of

cercariae is possible, it is unlikely given their small size and translucent colouration.

Evaluation of olfactory cues is a further possibility, although Poulin *et al.* (1999)

determined that the odour of *Diplostomum spathaceum* cercariae was not detectable by

naïve rainbow trout. Moreover, avoidance based upon visual and/or olfactory cues are

unlikely to explain my results since the minnows used in the experiment had no prior exposure and thus, could not associate the presence of cercariae in the water column to the negative outcomes of infection. Detection following the active penetration of cercariae is a further possibility. During some trials, I observed the characteristic 'shaking' and 'twitching' responses that some hosts demonstrate in response to cercariae penetration (Thiemann & Wassersug, 2000) indicating discomfort for the host. Detection of this sort could result in a generalized stress response, such as area aversion or shoaling.

One possible indirect mechanism of parasite detection by minnows is the 'alarm' cues released from damaged epidermal tissue of conspecifics that have recently been penetrated by cercariae. As members of the Superorder Ostariophysi, fathead minnows possess epidermal club cells that when damaged emit an 'alarm' cue causing conspecifics within sensory range to react with several avoidance behaviours including shoaling (review by Smith, 1992; Wisenden, 2000). Cercariae of *Diplostomum spathaceum* cause sufficient damage to the epidermis of rainbow trout to release these 'alarm' cues (Poulin *et al*., 1999) and thus, it is possible that the shoaling response observed in this study results from the detection of damaged club cells (or their products) and not a direct aversion to the cercariae themselves. An alternative indirect mechanism of parasite detection is information sharing between conspecifics of the potential risk of infection. Thus, minnows may use information provided by nearby parasitized conspecifics to trigger a shoaling response to potentially evade contact with cercariae. Unfortunately, I cannot distinguish the various potential mechanisms leading to the shoaling response of fathead minnows exposed to *Ornithodiplostomum* sp. cercariae. Utilization of a similar

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experimental protocol, but with the incorporation of exposure trials involving cercariae-conditioned water, or dead cercariae (e.g., Chapter 3), or species of cercariae that do not infect minnows would be useful.

Support for the shoaling hypothesis requires that individual minnows in the artificial shoals contain fewer metacercariae than minnows outside shoals. Although the results described in Figures 2.4 and 2.5 indicate a tendency for reduced exposure in shoaling fish, the differences were not statistically significant. However, before the shoaling hypothesis can be discounted, two important caveats should be considered. First, because I could not control the numbers of cercariae released from individual snails, rates of exposure were highly variable between individual ponds. Overall, individual minnows were exposed to between approximately 20 and 400 cercariae, despite efforts to control for features such as snail size and age. This magnitude of variation within and between ponds placed severe restrictions on my ability to detect a significant effect. Overall however, for metacercariae counts between individual ponds, there was no correlation between mean metacercariae intensity and variation in intensity. This indicates that even in ponds containing minnows with relatively few metacercariae, variation in metacercariae counts was as high in 'low-intensity' ponds as in 'high-intensity' ponds. Since there was no tendency for differences in shoal versus non-shoal minnows to be greater in 'low-intensity' ponds, it suggests that an overall masking effect of high cercariae numbers is not important.

A second important caveat is the artificial nature of the cages, especially in the context of interference with normal avoidance behaviours, or even normal foraging behaviours (Lafferty, 1999) that might interfere with the normal infection process. For example, rainbow trout (*Oncorhynchus mykiss*) prefer to move away from refugia containing cercariae of the trematode *Diplostomum spathaceum* and delaying an avoidance response is positively correlated with infection (Karvonen *et al.*, 2004). Fathead minnows may prefer a similar response in the wild, yet caging fish would restrict their ability to do so. Moreover, the movement of fish within cages is highly artificial and may also influence detection and contact by cercariae. Cercariae use abiotic cues created by fish hosts such as turbulence and shadows as reliable indicators of host location (Haas, 1994). It is conceivable that the constrained nature of the cages disrupts these typical cues and impairs the ability of cercariae to locate their intermediate hosts.

Taken together these results suggest that in a lab environment, shoaling is a preferred response of minnows facing infection yet associating with shoal-mates in semi-natural conditions does not benefit individuals. However, it is important to note that shoaling alone might not be the answer. For example, reduced movement is another behavioural strategy observed in some aquatic hosts exposed to trematode cercariae (Theimann & Wassersug, 2000). A reduction in activity may be beneficial to minnows exposed to cercariae because it forces cercariae, which are slow moving (Haas, 1994), to actively seek out their intermediate host. In a preliminary experiment comparing infection of minnows in cages versus non-caged controls, non-caged minnows had on average 4X more metacercariae than their caged counterparts. Non-caged minnows

could roam freely through their containers and as a result, likely came in contact with more cercariae. By restricting minnows to cages, the antiparasite benefits associated with shoaling may have been masked by the benefits of decreased host activity. Moreover, shoaling is often associated with a decrease in activity (Mathis & Smith, 1993) and therefore shoaling may provide antiparasite benefits in the form of reduced movement. Unfortunately, I could not distinguish between the effects of cages versus reduced activity on parasite infectivity. Thus, studies that combine tests of shoaling and inactivity might be the way forward.

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Table 2.1. Summary statistics for all 'shoal' and 'non-shoal' caged replicates.

		Shoal					Non-shoal					
Pond	n	mean ± SD	range	median	CV (%)	n	mean	range	median	CV (%)	Wilcoxon p	Pearson's r *
1	4	62.3 ± 9.5	51-74	62	15.3	6	97.2 ± 49.8	49-189	87	51.3	0.1995	0.0557
2	4	161.5 ± 77.5	66-254	163	48.0	3	253.3 ± 91.8	179-356	225	36.3	0.2159	0.5318
3	5	75.0 ± 22.3	47-108	69	29.8	6	108.5 ± 35.8	60-167	109.5	32.9	0.5822	0.3814
4	4	89.0 ± 46.3	51-151	76.5	52.1	3	70.0 ± 44.8	43-122	46	64.0	0.3768	0.7240
5	5	129.8 ± 32.4	99-176	129	25.0	4	166.3 ± 49.1	102-221	171	29.5	0.2187	0.4655
6	5	169.4 ± 65.4	75-225	196	38.6	5	105.4 ± 31.5	63-133	103	29.9	0.2101	0.0490
7	6	64.7 ± 18.8	31-83	67	29.1	5	79.2 ± 17.3	61-101	85	21.8	0.2353	0.4342
8	6	75.0 ± 23.9	38-99	79	31.9	6	73.3 ± 38.0	20-123	70	51.9	1.0000	0.4962
Average	39	101.5 ± 55.4	31-254	82	54.6	38	111.4 ± 64.1	20-356	101.5	57.6	0.3864	0.1020

* Correlation coefficient for association between minnow length and metacercariae intensity (no significant correlations)
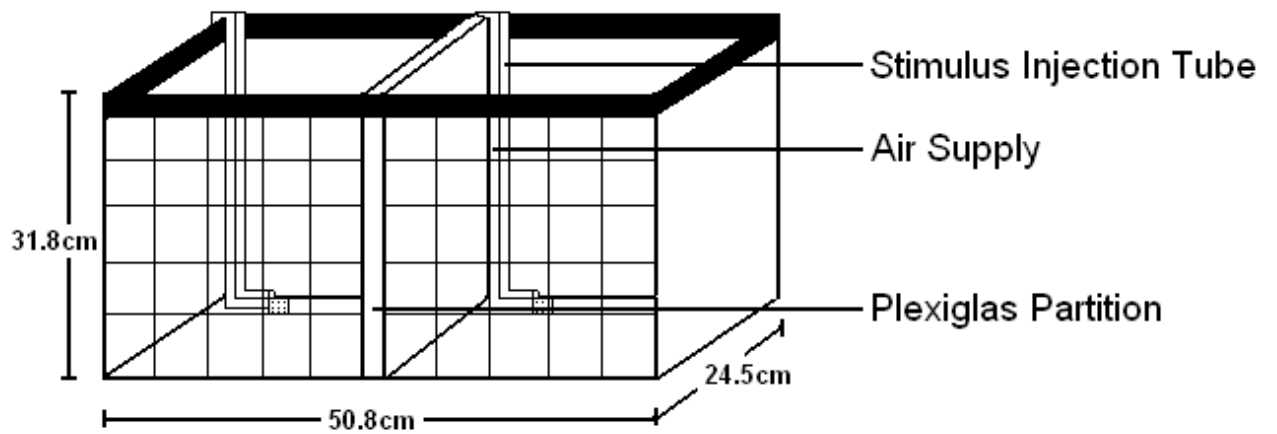
Figure 2.1. Scientific representation of experimental aquaria setup for shoal cohesion trials.
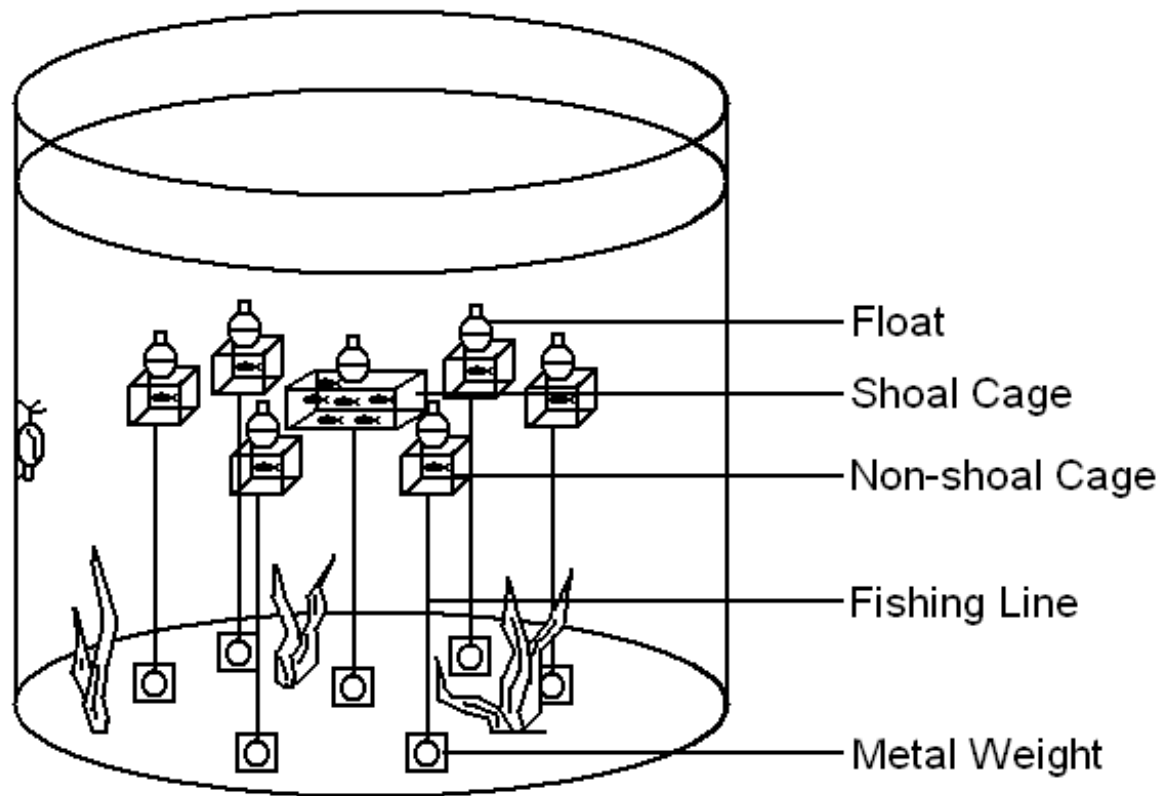
Figure 2.2. Scientific representation of mesocosm setup up for test of role of shoaling in defense against trematode cercariae.
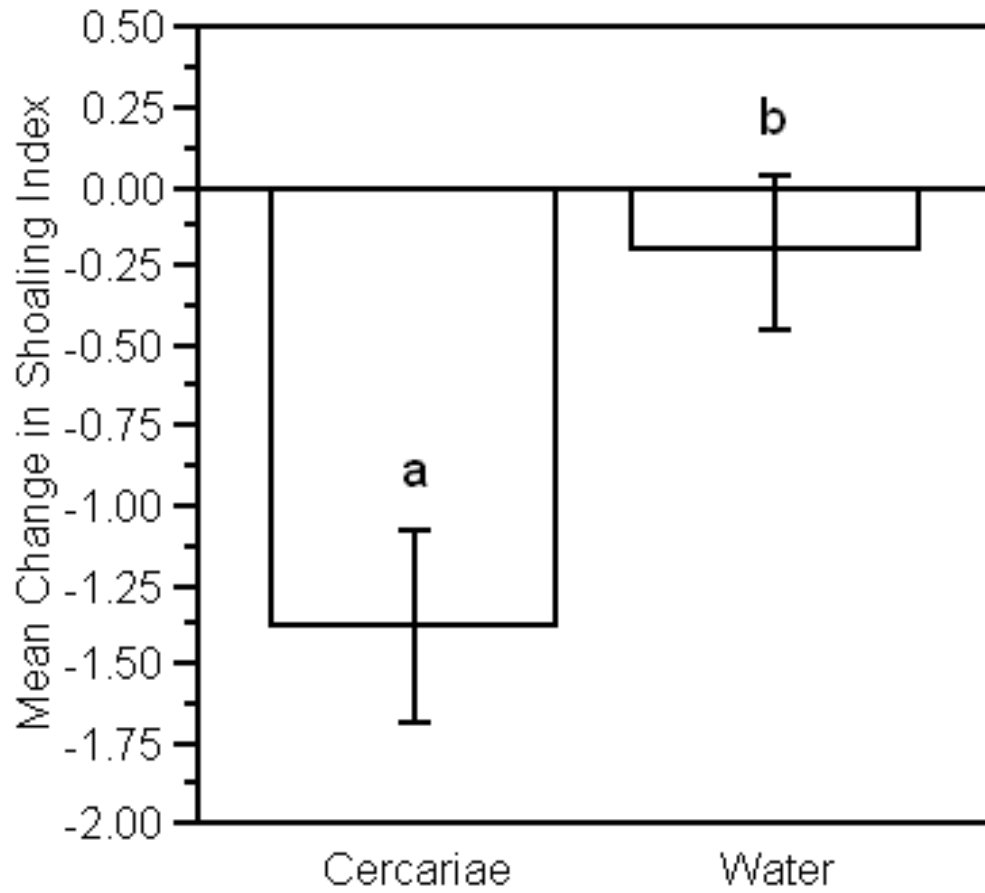
Figure 2.3. Mean (± SE) change in cohesion for fathead minnows exposed to cercariae and fathead minnows exposed to dechlorinated tap water. Different letters above bars indicate statistical significance (p < 0.05).
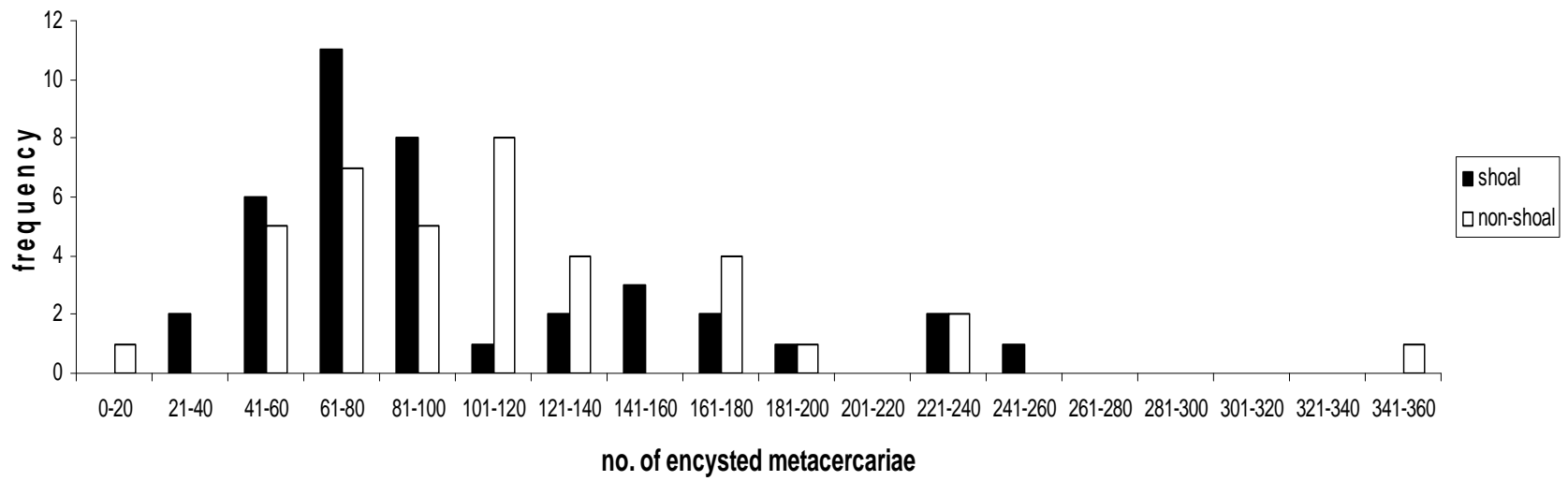
Figure 2.4. Frequency distribution of encysted metacercariae found in 'shoal' and 'non-shoal' configurations.
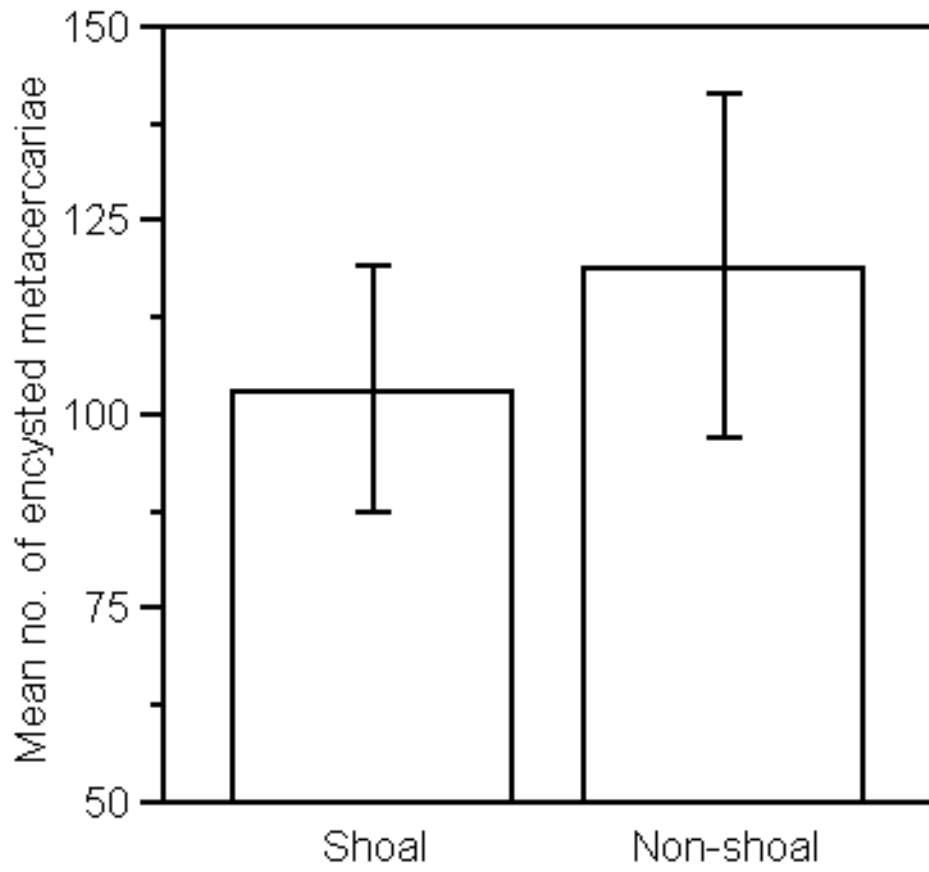
Figure 2.5. Mean (± SE) metacercariae intensity in fathead minnows constricted in shoaling and non-shoaling configurations.

Chapter 3. The role of experience in acquired recognition of parasite risk by fathead minnows exposed to trematode cercariae

ABSTRACT

Because parasites impose negative fitness consequences on hosts, hosts should be selected to detect and recognize parasite risk and evolve behavioural responses that minimize exposure. I evaluated the ability of fathead minnows to detect, recognize and avoid cercariae of the trematode *Ornithodiplostomum* sp. via olfactory and visual indicators. Cercariae of this species infect minnows by penetrating the skin and encysting within the body cavity. In the experiment, parasite-naïve minnows showed no evidence of innate recognition or avoidance of cercariae. However, after a single exposure to cercariae, fish responded to chemical and visual cues of thawed (dead) cercariae with a reduction in activity. Active fish probably encounter more cercariae and thus suffer higher rates of exposure than inactive fish. Thus, these data indicate that experienced minnows associate parasite risk with novel chemical and visual cues that later trigger parasite avoidance behaviour. These results open new avenues of research in behavioural ecology to study risk-sensitive decision making when the risk is parasitism, when the risk is predation, and when the two forms of risk co-occur.

INTRODUCTION

The evolutionary origin of antiparasite behaviours may be difficult to distinguish from the evolutionary origin of antipredator behaviours (Poulin *et al*., 1999). Indeed, generic behavioural reactions to risk may serve to reduce exposure to both parasites and predators. For example, predators often locate their host or prey by detecting motion (Lima & Dill, 1990). Similarly, the infective stages of many ecto- and endoparasites detect their host via visual, chemical and tactile cues that indicate host motility (Haas, 1994; Poulin *et al*., 1999). Not surprisingly, reduced activity is a common counter-strategy used by hosts and prey to reduce risk of detection by parasites and predators (Lawrence & Smith, 1989; Lima & Dill, 1990; Poulin *et al*., 1991; Chivers & Smith, 1998; Thiemann & Wassersug, 2000).

As with predator avoidance, parasite avoidance begins with detection of the parasite or indicators of parasitism risk (Lima & Dill, 1990; Smith, 1992; Hart, 1994; Kiesecker *et al*., 1999; Kiesecker & Skelly, 2000; Wisenden *et al*., in press). In aquatic ecosystems, chemical cues represent reliable public information about predation risk (Dodson *et al*., 1994; Kats & Dill, 1998; Wisenden, 2003; Wisenden & Chivers, 2006). These chemical cues can elicit evasive responses innately (i.e., without prior experience) or evasive responses can be acquired by associating a novel neutral stimulus with a stimulus known to be correlated with risk (Wisenden, 2003). Injury-released chemical cues reliably provide public information about the presence of an actively foraging predator, that in turn afford nearby prey the opportunity to associate the appearance and chemical signature of a novel predator with predation risk (Chivers & Smith, 1998). A

remarkable property of this type of learning is that one event is sufficient for near-permanent recognition of a novel predator with risk (Suboski, 1990; Chivers & Smith, 1994; see Brown, 2003 for review). In future encounters with the predator, indirect indicators of risk are sufficient to elicit an evasive behavioural response.

Given that sophisticated behavioural learning paradigms exist to counter one form of threat, it is plausible that learning can serve as a mechanism for detection and avoidance of parasite threat. Here, I test if parasite-naïve fathead minnows (*Pimephales promelas*) innately recognize and avoid parasites, and if not, if they can do so after given the opportunity to associate parasite risk with olfactory and visual indicators of parasites. This learning paradigm would allow minnows to adapt and respond to exposure risk that changes through host ontogeny, and ecological time and space. Fathead minnows present an ideal test organism for acquired recognition of parasite risk because they have been used for many studies in learned associations of predation risk (Chivers & Smith, 1998). Moreover, natural populations of minnows typically contain individuals that harbor many different types of parasites, some of which are amenable to experimental manipulation (e.g., Shirakashi & Goater, 2005 and references therein). Therefore, the aim of this study was to determine if minnows 1) have innate recognition and avoidance of *Ornithodiplostomum* sp. (Trematoda: Diplostomatidae) cercariae and 2) if behavioural responses that likely reduce risk of exposure to cercariae are acquired by previous exposure to parasites.

METHODS

Host/parasite system

Approximately 30 fathead minnows were obtained from the Environmental Protection Agency facility in Duluth, Minnesota, USA. These fish were lab-reared and thus parasite-free. They were purchased as 30-d-old juveniles and they were 50-d-old when used in the experiment. Fish were housed in a 185-L tank at 18° C, with a 12:12 L:D photoperiod. Fish were fed daily with commercial flake food. The mean ($\pm$ SD) total length of minnows used in the study was 2.87 ± 0.07 cm.

Ornithodiplostomum sp**.** is congeneric with *O. ptychocheilus*, a well known trematode of fathead minnows (e.g., Shirakashi & Goater, 2005). In lakes and ponds in Alberta, *Ornithodiplostomum* sp. is sympatric with and typically co-occurs with *O. ptychocheilus* in the same individual minnows (Sandland *et al.*, 2001). *Ornithodiplostomum ptychocheilus* encysts within the optic lobes of minnows, whereas *Ornithodiplostomum* sp. encysts in the body cavity. Their life cycles are very similar, involving physid snails as first intermediate host, fathead minnows as second intermediate host, and fish-eating birds as final host. As for most aquatic cercariae, infection of the second intermediate host occurs via penetration.

Experimental infections

For experimental infections involving cercariae of *Ornithodiplostomum* sp., I followed the methods of Sandland and Goater (2000) for *O. ptychocheilus*. Eight, 1-d-old chickens

were fed metacercariae collected in May, 2007 from the infected viscera of wild minnows obtained from Gold Spring Lake, southern Alberta, Canada. Trematode eggs were collected from chick faeces 5-d post-infection and F1 pond snails (*Physa gyrina*), lab-reared from parents collected in June of 2007, were exposed to the resulting miracidia. Snails were housed in 2-L plastic containers at 18° C, with a 12:12 L:D photoperiod and were fed romaine lettuce daily. To obtain cercariae, nine infected snails were isolated in small vials with dechlorinated tap water for 2-hr under direct artificial light. Cercarial counts in three 1-mL aliquots were averaged to estimate the volume of water needed to obtain a dose of 50 and 150 cercariae.

Conditioning and test stimuli

Fish were individually placed in 118-mL plastic cups with 30-ml of dechlorinated tap water. There were 3 conditioning treatment groups with 8 fish per group: (1) water control (2) parasite-naïve minnows, and (3) parasite-experienced minnows. Fish in the water control and naïve treatment groups each received 10-mL of blank dechlorinated water while fish in the experienced treatment each received 10-mL of dechlorinated water containing 50 live cercariae (Table 3.1). All fish were left in the plastic cups and not disturbed for two hours and then placed in 37-L aquaria following exposures based on treatment. After 2-d, fish were transferred to test aquaria.

Test stimuli for the trials were made by preparing 10-mL volumes that contained dechlorinated tap water only (water control group) or 10-mL of water with 150 cercariae

(used for parasite-naïve and experienced treatment groups) (Table 3.1). Test cues were

frozen at -20 ºC until needed. Freezing is a recognized method for preserving the cues of

cercariae (Bourns, 1963). Because freezing killed the cercariae, behavioural responses to

this cue could only be due to innate (naïve group) or learned (experienced group)

recognition of olfactory and/or visual cues of cercariae, and not from direct attacks.

Four-wk following the trials, infected fish were humanely killed with an overdose

of methane tricaine sulphonate and necropsied to evaluate the numbers of metacercariae

in exposed fish.

Test for behavioural response to cercariae

A grid (5 cm x 5 cm) was drawn on the end panel of 24, 37-L experimental tanks using a

black pen. The remaining sides were painted blue to prevent visual contact between

neighboring tanks. A thin plastic tube provided compressed air to a Dirt-magnet® sponge

filter placed in the center of each tank. A second airline hose was wedged into the rigid

plastic lift tube of the filter. This second hose was used to surreptitiously inject stimuli

into the test aquarium. The turbulence of the air flow masked any pressure changes

associated with cue injection, and water currents created by the filter quickly dispersed

test stimuli throughout the test aquarium. The stimulus hose was taped to the shelving to

prevent the hose from obstructing the view of the tank and to anchor the hose so that hose

movement would not disturb the fish during stimulus injection. Injection hoses were

about 2 m in length and allowed experimenters to conveniently introduce stimuli without disturbing the test subject.

Experimental protocol

Conditioned fish were placed individually into 37-L experimental tanks and allowed to acclimate for 24-h. They were fed commercial flake food before trials began. The stimulus hose for each experimental tank was rinsed by twice withdrawing and discarding 60-mL of tank water. A third 60-mL of tank water was retained to be later used to flush test stimuli into the test aquarium. Each fish was observed for 11-min: 5-min prestimulus behaviour, followed immediately by 1-min of stimulus injection during which time no behaviour was recorded, followed immediately by 5-min of post stimulus behavior. I recorded activity and vertical distribution because reduction in activity and movement toward the bottom are well documented behavioural responses to predation risk (Lawrence & Smith, 1989; Chivers & Smith, 1998) and parasite risk (Poulin *et al*., 1991; Thiemann & Wassersug, 2000). I recorded the depth of the fish every 15-s (score ranged from 1-5, with 1 for the surface row and 5 for the bottom row). Activity was scored as the total number of grid lines crossed in 5-min.

Analyses

All data were assessed for normality prior to analysis using the Shapiro-Wilk test for normality. Host lengths were compared using a one-way ANOVA. Activity levels prior to the addition of stimuli were compared with a one-way ANOVA. The purpose of this

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was to separate out any possible effects that prior infection would have on host activity. Following the protocol of Wisenden and Harter (2001), the overall change in host activity and vertical distribution was determined by subtracting the pre-stimulus data from the post-stimulus data. Changes in activity and vertical distribution were compared with one-way ANOVA's. For post-hoc comparisons, a Tukey Kramer test was performed.

RESULTS

Fish lengths did not differ among treatment groups (one-way ANOVA: $F_{2, 21}$ = 10.38, p = 0.36) and encysted metacercariae were present in all fish in the parasite-experienced treatment (mean = 24.3, SD = 6.3). Prior to the addition of stimuli, fish activity was normally distributed (Shapiro-Wilk Test, W = 0.92, p = 0.07) and did not significantly differ between treatments (one-way ANOVA $F_{2, 21}$ = 0.55, p = 0.59).

Change (poststimulus-prestimulus) in fish activity and change in vertical distribution conformed to a normal distribution (Shapiro-Wilk Test: Δ activity, W = 0.92, p = 0.07; Δ vertical distribution, W = 0.92, p = 0.06). Change in activity was significantly affected by treatment (one-way ANOVA $F_{2, 21}$ = 5.27, p = 0.014). Experienced fish reduced activity in test trials, whereas naïve fish and water control fish increased activity (Fig. 3.1). Tukey post-hoc pairwise comparisons showed that experienced fish reduced their activity significantly more than water controls. There was no effect of treatment on change in vertical distribution (one-way ANOVA $F_{2, 21}$ = 0.92, p = 0.41; Fig. 3.2).

DISCUSSION

This study is the first to demonstrate that hosts can acquire behavioural avoidance of parasite risk through experience. Parasite avoidance behaviours are well documented and have been previously observed in other fish/parasite systems (Poulin & Fitzgerald, 1989; Karvonen *et al*., 2004), but the mechanisms for detection (specifically for the avoidance of trematode cercariae) are rarely understood. For example, rainbow trout avoid cercariae by moving away from the source, but whether or not this is a result of detecting the cercariae before exposure via visual/olfactory cues or a response to direct penetration by cercariae is unknown (Karvonen *et al*., 2004). My results are the first to show fish can detect sensory cues associated with trematode cercariae. I showed that a single exposure to cercariae is sufficient to alter future responses of fathead minnows to chemical and visual indicators of parasite risk. Behavioural response was manifest as a reduction in activity, a behavioural strategy that has been shown to reduce the risk of parasite exposure in other studies (Poulin *et al*., 1991; Thiemann & Wassersug, 2000). Parasite-naïve fish did not reduce activity in response to thawed (dead) cercariae and their behavioural response did not differ from control fish. This latter finding suggests that naïve minnows do not innately recognize the general odour or sight of cercariae as indictors of parasite risk. These results concur with those of Poulin *et al*. (1999) and Thiemann and Wassersug (2000) in that behavioural responses to parasite risk are similar to behavioural responses to predator risk.

One alternative explanation for these data is that initial infection in experienced fish reduced activity levels relative to naïve fish. Metacercariae-induced changes in fish

behaviour are well known, and include both increases and decreases in the activity of infected individuals (reviewed by Barber *et al.*, 2000). However, infected fish did not differ in activity prior to the addition of stimuli compared to non-infected fish and therefore, the marked reduction in activity observed in experienced minnows is likely a response to the addition of thawed cercariae and not a byproduct of infection. Still, I cannot rule out the possibility that metacercariae in experienced fish caused subtle changes in host activity compared to uninfected controls.

Pairwise comparison between naïve and experienced fish in their reduction in activity failed to surpass the threshold of statistical significance. This is in part due to small sample size, but due in part also to decreased activity by some parasite-naïve minnows. No water control fish showed a decrease in activity. From these data I conclude that parasite-naïve minnows detected a novel odour in the test stimulus and were cautious, but not fearful. Because there was no evidence of an increase in activity (compared to water control fish) I conclude that parasite-naïve minnow*s* did not perceive the cercariae as a source of food.

Wild minnows in natural populations should favour moving to the substrate to avoid parasites because trematode cercariae of a closely related species (*Diplostomum spathaceum*) tend to position themselves close to the water surface (Haas, 1994). This would be a second parallel between antipredator and antiparasite behavioural responses. Movement toward the substratum is a behavioural response to risk of predation that has been recorded in minnows exposed to predator cues and chemical alarm cues in skin

extract (Lawrence & Smith, 1989; Chivers & Smith, 1998). However, in my study, there was no effect of treatment on change in vertical distribution. Because fish in my study were lab-reared, even parasite 'experienced' fish had no basis for associating parasite risk with a certain stratum of the water column. Moreover, the small size of tanks used in this experiment and the method of stimulus dispersion evenly distributed cercariae throughout the water column making it impossible to avoid cercariae by changing depth.

Aquatic, free-living cercarial stages of trematodes use a variety of environmental cues to indicate the presence of a potential host (Haas, 1994). Specifically, turbulence, caused by host activity, is a good indicator of host location (Haas, 1994). Therefore, reduced activity in the presence of visual and/or olfactory indicators of free-swimming cercariae leads to reduced exposure to parasite risk (Poulin *et al*., 1999). Concomittently, reduction in activity reduces exposure to predation risk (Mathis & Smith, 1993). Thiemann and Wassersug (2000) showed that tadpoles of *Rana sylvatica* and *R. clamitans* evaluate the risk of parasitism and predation independently and respond by additively adjusting the intensity of a single behavioural response, reduction in activity. Activity reduction was in proportion to the degree of risk. Because predators pose a greater threat to tadpole survival than parasites, the degree of reduction of tadpole activity was reduced in the presence of parasites yet reduced significantly further when tadpoles were also confronted with predators (Thiemann & Wassersug, 2000). Reduction in tadpole activity is an adaptive response to parasitism that parallels a similar, yet stronger response to predation.

I argue that predation risk and parasite risk share certain features that make activity reduction a behavioural stratagem that reduces exposure to both risks. However, predation risk differs from parasite risk in that predation is an absolute loss of fitness whereas fitness consequences of parasitism may be negligible and/or delayed past the reproductive age. Behavioural trade-offs between parasite risk and other behaviours (e.g., foraging, reproduction) are likely to differ in scale and nature from trade-offs between those same behaviours and predation risk. Parasite threat-sensitive behavioural decision-making is a ripe area for future study.

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Table 3.1. Test stimuli presented for conditioning and test trials (n = 8 per treatment)

Treatment	Conditioning stimulus	Test stimulus
Water Control	10-ml dechlorinated water	10-ml dechlorinated water
Naïve	10-ml dechlorinated water	10-ml with 150 thawed (dead) cercariae
Experienced	10-ml with 50 live cercariae	10-ml with 150 thawed (dead) cercariae
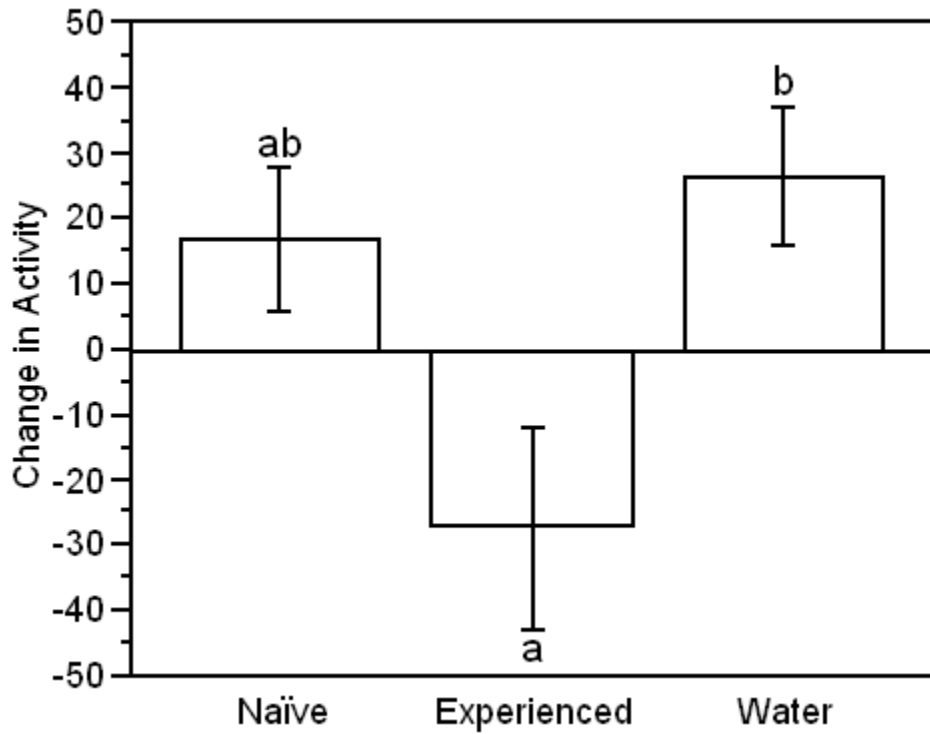
Figure 3.1. Mean (± SE) change in activity (number of grid lines crossed) for 1) cercariae-naïve minnows in response to the odour and sight of dead cercariae, 2) cercariae-experienced minnows in response to the odour and sight of dead cercariae or 3) cercariae-naïve minnows in response to blank water (control). Shared letters above bars indicate no difference ($p < 0.05$) in Tukey post-hoc pairwise comparisons.
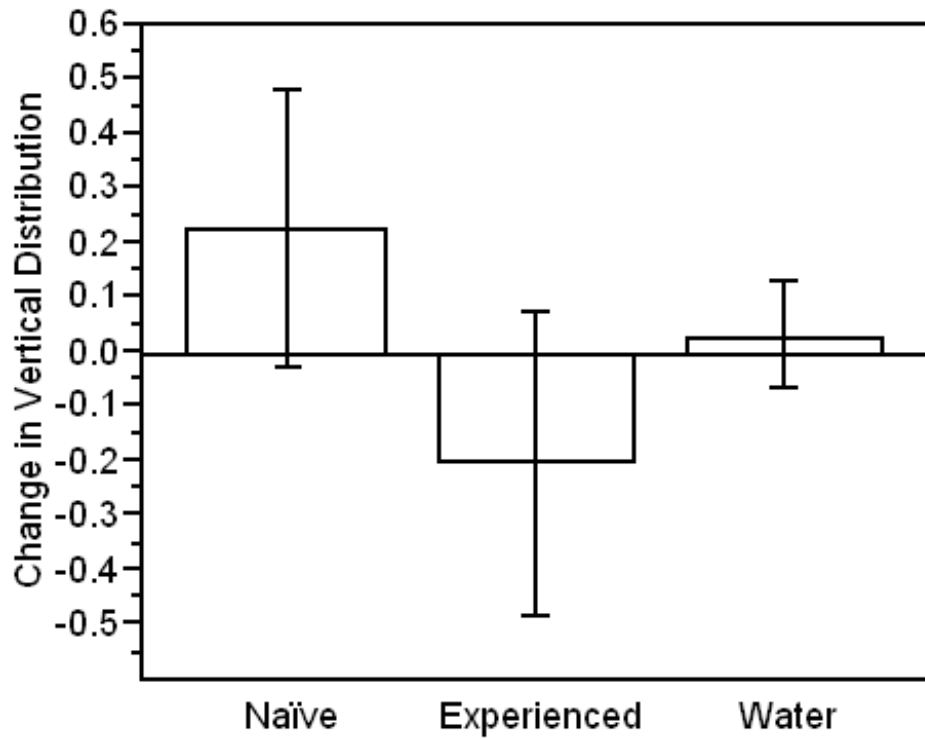
Figure 3.2. Mean (± SE) change in vertical distribution (grid line depth) for 1) cercariae-naïve minnows in response to the odour and sight of dead cercariae, 2) cercariae-experienced minnows in response to the odour and sight of dead cercariae or 3) cercariae-naïve minnows in response to blank water (control).

Chapter 4. The protective role of club cells in fathead minnows exposed to trematode cercariae: a test of the antipathogen hypothesis

ABSTRACT

Many aquatic organisms release chemical alarm cues when epidermal tissue is damaged. Well-studied examples are the epidermal club cells of fishes in the Superorder Ostariophysi. However, explaining the evolution of club cells by selection from antipredator benefits relies on unlikely scenarios or group selection, leading to the development of several alternative hypotheses. Here, I provide some initial tests of the hypothesis that club cells serve an antipathogenic function. Cercariae of the trematode *Ornithodiplostomum* sp. were 18.1% and 17.0% less successful at encysting as metacercariae when initially immersed in a high concentration of fathead minnow skin extract, containing club cells, compared to a low concentration and water controls, respectively. I repeated this first experiment with the addition of a treatment containing skin extract from mollies; a non-ostariophysan that lacks club cells and I found that cercariae immersed in molly and minnow skin extract were 68% and 61% less successful at reaching encystment compared to water controls, respectively. These results indicate that club cells are not the active component in minnow skin reducing trematode infectivity. Previous findings also suggest that fathead minnows invest in higher densities of club cells following infection by trematode cercariae. Here, I found no difference in club cell density between minnows exposed to 2 doses (high and low) of *Ornithodiplostomum* sp. cercariae and water controls, suggesting that this is not a universal response to all skin-penetrating trematodes in minnows. Taken together, these

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results do not support the antipathogen hypothesis for the evolution of club cells in fish

responding to skin-penetrating trematodes.

INTRODUCTION

Parasites impose a wide variety of costs on their hosts. Given the magnitude of such costs, parasite-mediated selection should favour host defenses that either limit exposure to parasites, or limit their negative effects (review in Chapter 1; see also Goater & Holmes, 1997). For hosts, limiting exposure to parasites can be achieved using avoidance behaviours following parasite detection (Hart, 1994). Studies focusing on host avoidance behaviours have become more commonplace yet despite recent attention, evidence remains weak (Hart, 1994; Moore, 2002; Wisenden *et al.*, In press). Exceptions include the avoidance of biting arthropods and fecal matter containing nematode larvae by grazing animals (Moore, 2002). A recent review by Wisenden *et al.* (In press) showed that solid evidence for behavioural avoidance in fish prior to exposure is limited to conspicuous and highly pathogenic infective stages of parasites. Thus, for many host/parasite interactions, hosts appear only poorly adapted to avoid even harmful parasites. Therefore, to reduce parasite-induced costs, post-exposure mechanisms should be under strong selection.

Various arms of the host immune system are obvious candidates for natural selection. However, components expressed by the vertebrate immune system as a response to parasite infection come at the expense of other important fitness traits such as growth and reproduction (Sheldon & Verhulst 1996; Zuk & Stoehr 2003). It makes sense that natural selection may prefer a more preventative approach to infection by defending against parasites at the point of contact or entry into the host, reducing associated costs of parasites at the site of infection. Epidermal immunity to the skin-penetrating infective

stage (schistosomula) of *Schistosoma mansoni* is a popular example of this idea (Wakelin, 1984). Similarly in fish hosts, the epidermis is a likely candidate for preventative immunity. Immune components found in fish mucus (produced by goblet cells located in the epidermis) have been shown to reduce the success of various pathogens at the site of infection (review by Shephard, 1994). Another well-studied cellular component of fish epidermis that may have possible immunity-based implications is the alarm substance cells, or club cells, found in fish from the Superorder Ostariophysi (comprising 74% of all freshwater fish species (Nelson, 1994)).

Smith (1982) and Magurran *et al*. (1996) first posited that club cells may contain antipathogenic agents and more recently, Chivers *et al*. (2007) provide empirical evidence in support of this hypothesis. It is well known that club cells serve as a mechanism for risk assessment used by fish to detect and avoid danger in the event a conspecific is attacked and injured by a predator (review by Smith, 1992). Investing in the production and maintenance of club cells is costly to host fish so a reciprocal benefit to the investor is necessary for club cells to be maintained over evolutionary time (Wisenden & Smith, 1997). However, the benefits remain unclear for the individual under predator attack and few data support the idea that club cells evolved primarily as a defense against predation (Mathis *et al*., 1995; Chivers *et al*., 1996). Since ostariophysan fish frequently host a wide variety of trematodes (Erasmus, 1972; Williams & Jones, 1994) that attach to or penetrate epidermal tissue, free-swimming penetrative life cycle stages (i.e., cercariae) seem a likely target of epidermal club cells should their primary function be antipathogenic.

Two indirect lines of evidence support the prediction of the antipathogen hypothesis of club cells for trematode infections of fish. First, most cercariae of fish have an obligate penetrative phase (they are only rarely ingested as part of the infection process) (Erasmus, 1972). Although cercariae vary widely in size across species, most cercariae are large relative to club cells and as such, likely pass through or disrupt these cells during penetration. Another indirect line of evidence is the chemical structure of club cells. The active chemical in club cells that elicits alarm reactions in conspecifics is a nitrogen oxide side group such as the one contained in hypoxanthine-3-*N*-oxide (Pfeiffer, 1985; Brown *et al.*, 2000). Nitrogen oxides play an important role in host resistance to the free-living stages of helminth parasites (James, 1991). Nitrogen oxides are effector molecules that specifically disrupt the larval stage (schistosomula) of *Schistosoma mansoni* by inhibiting metabolic activity (James & Hibbs, 1990). Schistosomula are similar to the migrating cercarial stage (diplostomula) used in this study (Erasmus, 1972) and therefore I predict that nitrogen oxides in club cells play a similar larvacidal role that may interfere with the development of metacercariae.

There are also 2 direct lines of evidence in support of the antipathogen hypothesis. In an experimental study, Chivers *et al.* (2007) showed that fish exposed to 70 trematode cercariae (*Telorchis* sp.) significantly increased their number of club cells compared to controls following infection. Thus, higher exposure leads to higher investment in club cells. Also, Poulin *et al.* (1999) showed that rainbow trout exposed to trematode cercariae (*Diplostomum* sp.) induced a fright reaction in naïve, unexposed conspecifics.

This result implies that trematode cercariae do indeed rupture club cells while penetrating the host epidermis and ultimately come in contact with the contents of these cells. However, whether these results are generalizable to other host/parasite systems is unknown.

Here I test two predictions of the antipathogen hypothesis. First, following similar methodologies of Chivers *et al*. (2007), I aim to determine if fish respond to trematode infection by increasing club cell investment. Second, if club cells contain antipathogenic properties, I aim to assess the infectivity of cercariae immersed in varying concentrations of skin extract (both with and without club cells). I utilize an experimental host/parasite interaction involving fathead minnows (*Pimephales promelas*) and one of their common skin-penetrating trematodes (*Ornithodiplostomum* sp.). I predict that alarm cell density will increase in the epidermis of fish exposed repeatedly to *Ornithodiplostomum* sp., similar to the results of Chivers *et al*. (2007), and predict that cercariae exposed to the contents of club cells will have reduced infectivity compared to controls.

METHODS

Host/parasite system

Fathead minnows serve as the second intermediate host of *Ornithodiplostomum* sp., an undescribed trematode that is closely related to *Ornithodiplostomum ptychocheilus* (Sandland *et al*., 2001). Metacercariae encyst in the body cavity of minnows and await predation by the definitive host, a fish-eating bird. Following ingestion, adult worms

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migrate to the gut of their definitive host and produce eggs that are shed in the bird's faeces. Eggs successfully deposited in water hatch into miracidia, a ciliated stage that actively seeks out and infects pond snails (*Physa* sp.). Following a 2 to 4-wk period of development, cercariae begin emerging from snails into the water column; this typically occurs between the months of June and October in natural conditions (Sandland *et al.*, 2001). Successful cercariae penetrate the epidermis of fathead minnows and then encyst as metacercariae following a 2 to 4-wk period.

Effect of minnow skin extract on cercariae infectivity – Experiment 1

Fathead minnows used in the experiment were obtained from U.S. EPA National Health and Environmental Effects Research Laboratory, Duluth, Minnesota, USA on 19-July, 2006. The minnows (30-d-old) were maintained in 4 separate 190-L tanks for 3-d before the experiment and were fed *ad lib* on Tetramin flake food.

Ornithodiplostomum sp. cercariae were obtained following the methods of Sandland and Goater (2000) for *O. ptychocheilus*. Eight, 1-d-old chickens were fed metacercariae from the viscera of wild fathead minnows collected in June from Gold Spring Lake, Alberta, Canada. Trematode eggs were collected from chick faeces 5-d post-infection. Miracidia that hatched from the eggs were pipetted into small vials containing laboratory-reared juvenile pond snails, *Physa gyrina*, measuring 3-5 mm in maximum length. Snails released cercariae at approximately 28-d post-exposure. To obtain cercariae, 12 infected snails were placed into a flask for 3-hr. The numbers of

cercariae in 3, 1-mL aliquot samples were counted and averaged to estimate the total numbers of cercariae released over 3-hr. I used this estimate to evaluate the volume of water containing 50 cercariae.

Skin extract was prepared following the methods of Wisenden *et al*. (1995). Wild adult female fathead minnows (mean = 5.74, SD = 1.07 cm, n = 55) were collected from Deming Lake, Minnesota, USA on 11-July, 2006. Each fish was euthanized by cervical dislocation. Skin fillets were removed from both sides of the fish, measured for area and then immersed in 100-mL of chilled dechlorinated tap water. The total area of skin collected from the 55 fish was 300 cm^2.

The batch of fillets was homogenized using a handheld blender and the solution filtered through a funnel packed with polyester fiber (Wisenden *et al*., 1995). 200-mL of dechlorinated tap water was added to the skin extract to rinse the blender, filter, and funnel. Dechlorinated tap water was added to bring the total volume of skin extract to 3-L. This served as a stock solution from which concentrations of 1.0 cm^2 skin/10-mL water (high concentration) and 0.1 cm^2 skin/10-mL of water (low concentration) were produced. Sixty, 40-mL aliquots of each concentration and blank dechlorinated tap water were frozen at -20°C.

The experiment was set up as 'concentration of extract' (low, high, water control) X 'time of cercarial immersion' (0-min, 30-min) factorial design. There were 20

replicates for each of the 6 treatments. Volumes containing 50 cercariae were pipetted into Petri dishes (diam = 8.5 cm) containing low, high, or control concentrations of skin extract. Half of the minnows assigned to a concentration treatment were added directly to individual Petri dishes prior to the addition of cercariae. This procedure meant that infection could occur instantaneously (0-min immersion). The other half were placed in the test solution 30-min after the addition of cercariae to see if prolonged exposure to skin extract reduced cercarial infectivity (30-min immersion). Petri dishes were arranged so that skin extract solutions (low, high, and control) were evenly distributed. After fish and cercariae had been added, the Petri dishes were covered for 2-hr and then the minnows were removed in the same order as added. It took approximately the same amount of time to add the cercariae as it did to remove the minnows.

Following exposure, minnows were placed into 6 separate 37-L tanks based on treatment (n = 20 minnows per tank). Minnows were fed tetramin flake food daily for 4-wk under a 12:12 L:D photoperiod to ensure that metacercariae would be fully developed at necropsy (Sandland & Goater, 2000). Minnows were euthanized with an overdose of methane tricaine sulphonate, dissected, and the numbers of metacercariae in the body cavity counted using standard methods.

Effect of minnow skin extract on cercariae infectivity – Experiment 2

A follow-up experiment was designed to replicate the salient features of the first experiment. The overall design was similar, with the following exceptions. First, I

incorporated a treatment involving skin extract removed from a Neotropical non-ostariophysan, mollies (*Poecilia sphenops*). The purpose of this addition was to isolate the effects of club cells, which are only present in ostariophysans.

For this experiment, the source of fathead minnows, their maintenance, the preparation of skin extract preparation, and the methods used for exposure was the same as those described above. Mollies were obtained from a commercial supplier and upon arrival, were immediately euthanized to prepare skin extract. Twenty-mL aliquots of each type of skin extract and dechlorinated water were pipetted separately into 105, 118-mL labeled plastic containers (n = 35 containers per treatment) with sealable lids and frozen at -20°C until needed.

The experiment was set up as a single factor factorial with 3 levels: water control, fathead minnow extract ($1cm^2$ of skin/10-mL), and molly skin extract ($1cm^2$ of skin/10-mL). On the day of exposure, the containers were removed from the freezer and thawed. One hundred cercariae were pipetted directly into each container and 30-min later, minnows were added. Containers were arranged so that all treatments were evenly distributed and minnows were assigned at random to containers. After exposure, all minnows were removed and separated by treatment and placed in 75-L tanks. Minnows were maintained on a daily flake food diet under a constant 12:12 L:D photoperiod for 5-wks. Minnows were then assessed for metacercarial intensity as described above.

Effect of cercariae on the density of epidermal club cells and epidermal thickness

If club cells provide protection against penetrating cercariae, individual fish should invest in more epidermal club cells following exposure. This experiment was designed to test this prediction. The source of fish, snails, and cercariae and the exposure protocol was the same as described for experiment 2.

A total of 90, 30-d-old minnows (average length = 29.72, SD = 2.08) were divided into 3 groups of 30 as follows. One group (high-dose group) was exposed to a total of 3 batches (1 batch per d) of 50, 2-hr old cercariae every 2-d commencing on 30-July, 2007 and ending on 3-August, 2007. A second group (low-dose group) was exposed on the same days to 10 cercariae; the third (control group) to water only. Fish were assigned at random to Petri dishes that contained 40-mL water and 10 or 50 cercariae. The exposure period was 2-hr.

Three-d following the third and final exposure, 20 fish from each treatment were euthanized and a section of tissue from the nape region was removed from each minnow, preserved in 10% buffered formalin and prepared for histological examination. The remaining 10 fish in each treatment were maintained on flake food for 4-wk to allow encystment of metacercariae to occur. Following the 4-wk period, fish were euthanized and necropsied for metacercariae intensity.

Tissue samples were sent to North Dakota State University Veterinary Diagnostic Laboratory where they were sectioned and transferred to slides. For each epidermal tissue sample, three paraffin-imbedded sections (7 μm thick) were removed and stained with Schiff's reagent and then counterstained with Lillie's haematoxylin (PAS-H). Each slide was photographed (Fig. 4.1). Three, 1 mm sections of each tissue sample were evaluated for the number of club cells. Epidermal thickness was also measured from the photograph and included the distance between the basement membrane of the epidermis and the outer surface of the epithelium (Wisenden & Smith, 1997). The 3 measurements of club cell density and epidermal thickness were averaged for each section to provide an estimate of an individual's investment in club cells. The observer was blind to all treatments.

Analyses

All data were tested for normality prior to analysis using the Shapiro-Wilk test for normality. Differences between host lengths for all experiments were compared using one-way ANOVA's. The effects of time and concentration on metacercarial intensity in the first experiment were analyzed using an ANCOVA with the total length of minnows as a covariate. Metacercarial intensity data were not normally distributed (Shapiro-Wilk test, $p < 0.0001$) for the second experiment and thus the differences between treatments were compared using a Kruskal-Wallis one-way ANOVA. Kruskal-Wallis multiple comparisons were performed following Siegel and Castellan (1988; pp 213-215). For the third experiment, alarm cell density and epidermal thickness were compared with one-way ANOVA's. Where appropriate, post-hoc comparisons of means between two samples were performed using a Tukey-Kramer test.

RESULTS

Experiment 1

Sixty-seven percent of the 120 minnows used in the experiment survived to 4-wk post-infection. Ten to 15 minnows survived within each treatment. All minnows that were dissected at the end of the experiment contained encysted metacercariae in the body cavity. There was no significant difference in host size among treatment groups, although minnows in the high concentration treatment tended to be slightly larger (one-way ANOVA, $F_{2, 77} = 2.80$; $p = 0.07$).

The mean number of encysted metacercariae was not significantly affected by the time X concentration interaction (two-way ANCOVA, $F_{2, 73} = 0.92$; $p = 0.40$). Thus, the main effects of time and concentration were considered independently. The concentration of skin extract significantly affected metacercarial intensity ($F_{2, 73} = 4.93$; $p = 0.01$, Fig. 4.2) but time did not ($F_{1, 73} = 2.81$; $p = 0.10$). For data pooled across the 2 time intervals, post hoc comparisons indicated that mean metacercarial intensity in minnows from the high-concentration extract was significantly lower than in both the control and low concentrations. There was a significant positive correlation between host length and the numbers of metacercariae ($F_{1, 73} = 11.69$; $p = 0.001$).

Experiment 2

Eighty seven percent of 105 minnows survived to 4-wk p.i. Overall, 29-32 minnows survived within each treatment. All surviving minnows were infected with encysted

metacercariae. Mean host length did not differ between the 3 host groups (one-way

ANOVA, $F_{2, 88} = 0.61$; $p = 0.55$)

The mean number of metacercariae was significantly affected by treatment

(Kruskal-Wallis one-way ANOVA, $KW = 60.93$, $df = 2$, $p < 0.001$, Fig. 4.3). Following

multiple comparisons, water controls differed from both skin extract treatments (minnow

and molly) in their number of encysted metacercariae but there was no difference in

metacercarial load between skin extract groups ($p = 0.05$).

Experiment 3

All fish exposed to cercariae were infected with metacercariae following the 4-wk

encystment period (mean # of cysts ± SD, low-dose group = 23.3 ± 7.3, n = 8, high-dose

group = 93.2 ± 20.7, n = 5). The average total length of minnows did not differ between

groups (one-way ANOVA, $F_{1, 57} = 0.39$, $p = 0.68$).

Cercarial dose did not significantly affect mean club cell density (one-way

ANCOVA, $F_{2, 57} = 0.37$, $p = 0.69$, Fig. 4.4) or epidermal thickness ($F_{2, 57} = 0.19$, $p = 0.83$,

Fig. 4.5).

DISCUSSION

Results from my study showed that cercaria infectivity was markedly reduced by momentary exposure to high concentrations of minnow skin extract. However, non-ostariophysan fish skin extract of the same concentration also reduced cercariae infectivity to approximately the same extent. Thus, some component of the epidermis of minnows and mollies reduces cercarial infectivity, but that component is likely not club cells. Moreover, fathead minnows did not increase their investment in club cells following a series of exposures to *Ornithodiplostomum* sp. cercariae. Neither of these results is consistent with the predictions of the antipathogen hypothesis proposed by Chivers *et al.* (2007).

The marked reduction in cercaria infectivity demonstrated in experiments 1 and 2 could result from some other component of fish epidermis that is shared between minnows and mollies. One possibility is that components of goblet cells act directly on penetrating cercariae to reduce infectivity. Goblet cell secretions contain a variety of antipathogenic compounds such as lysozymes, complement components, lectins, proteolytic enzymes, and immunoglobulins (reviews by Shephard, 1994; Buchmann & Brescianni, 1998). These secretions, alone and in combination, have been shown to protect fish from parasitic fungi, bacteria and monogenean trematodes (review by Shephard, 1994; Buchmann, 1999). Fathead minnows contain large numbers of these cells (Wisenden & Smith, 1997), but their functional role as a defense against parasites has not been evaluated and merits further investigation. Because all fish possess goblet cells within their epidermis (Shephard, 1994), components in fish mucus most likely

serve non-specific antipathogenic functions that protect fish from trematode cercariae similar to monogeneans (Buchmann, 1999). However, the specific stage of the infection process affected is unknown. Conceivable stages of cercarial infection that could be impeded include: attachment to the host, penetration through the epidermis, migration within the host, and the development into metacercariae (Haas, 1994). More than one, including any combination, of these stages may have been disrupted.

An alternative explanation is that a component of skin extract interferes with chemo-orientated host-finding behaviours of cercariae (see review by Haas, 1994). For example, cercariae of the trematode *Cryptocotyle lingua* greatly increase their activity and penetration behaviour in the presence of host skin extracts (Chapman, 1974). Thus, the skin extract (excess host cues) used in my study may have weakened the sensory ability of cercariae by masking the location of the fish hosts within the small confinements, even despite ample time allotted for exposure. However, Haas (1994) predicts that the observed increase in swimming activity of cercariae in host skin extract should improve the chance of host contact resulting in a greater rate of transmission, opposite to my findings. Moreover, cercariae do not rely solely on chemo-orientation for host location and could have used other cues (i.e., turbulence) to seek out their hosts (Haas, 1994).

The results of experiment 3 contrast those reported by Chivers *et al*. (2007), involving another skin-penetrating trematode of fathead minnows. In their study, minnows repeatedly exposed to *Telorchis* sp. cercariae had 16% more club cells in their

epidermis than those exposed to water controls, whereas minnows in my study did not

respond to infection by investing in more club cells. In addition, fish exposed to the

highest number of cercariae had the lowest number of club cells per millimeter, a trend

opposite of the direction reported by Chivers *et al*. (2007).

One explanation for my negative findings is that cercariae of *Ornithodiplostomum*

sp. may penetrate the host at an epidermal site other than the nape. The congener,

Ornithodiplostomum ptychocheilus, can penetrate the epidermis anywhere along the body

but some digenean trematodes prefer more specific locations for penetration. For

example, cercariae of the trematode *Diplostomum spathaceum* (closely related to

Ornithodiplostomum sp.) prefer to penetrate the gill chamber region and the connective

tissue of fin peduncles of rainbow trout (Ratanarat-Brockelman, 1974). Therefore, club

cell investment following infection could be site-specific and I may have overlooked the

appropriate area responding to infection. The nape was chosen to replicate Chivers *et al*.

(2007) with consistency and the concept of site-specific club cell investment is a novel

and untested idea.

Overall, the results provide little evidence in support of the antipathogen

hypothesis explaining the evolution of club cells. Because trematode cercariae represent

only a proportion of the parasites and pathogens that infect fish, it is too early to make

generalizations. However, the antipathogen hypothesis does not seem to hold true for

natural, long-standing cercariae/host interactions such as *Ornithodiplostomum* sp. in

fathead minnows.

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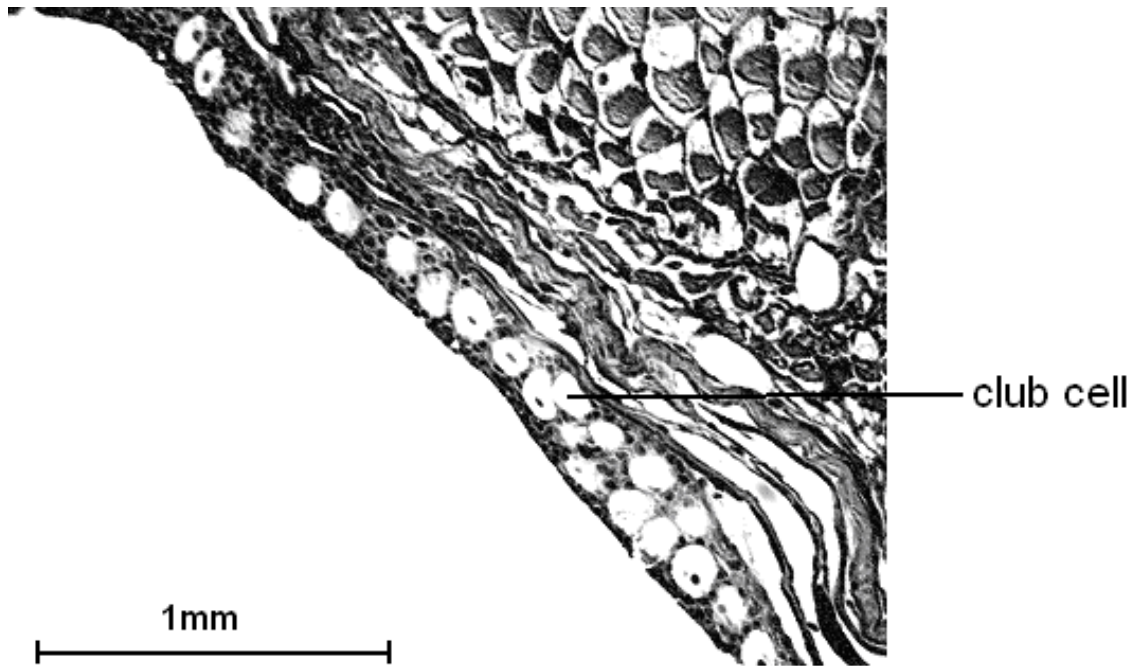
Figure 4.1. Digital photograph of epidermal cross-section (nape region) of a fathead minnow at 400X magnification. Each section was stained with Schiff's reagent and then counterstained with Lillie's haematoxylin (PAS-H). Clear cells denote club cells and dark cells denote mucus cells.
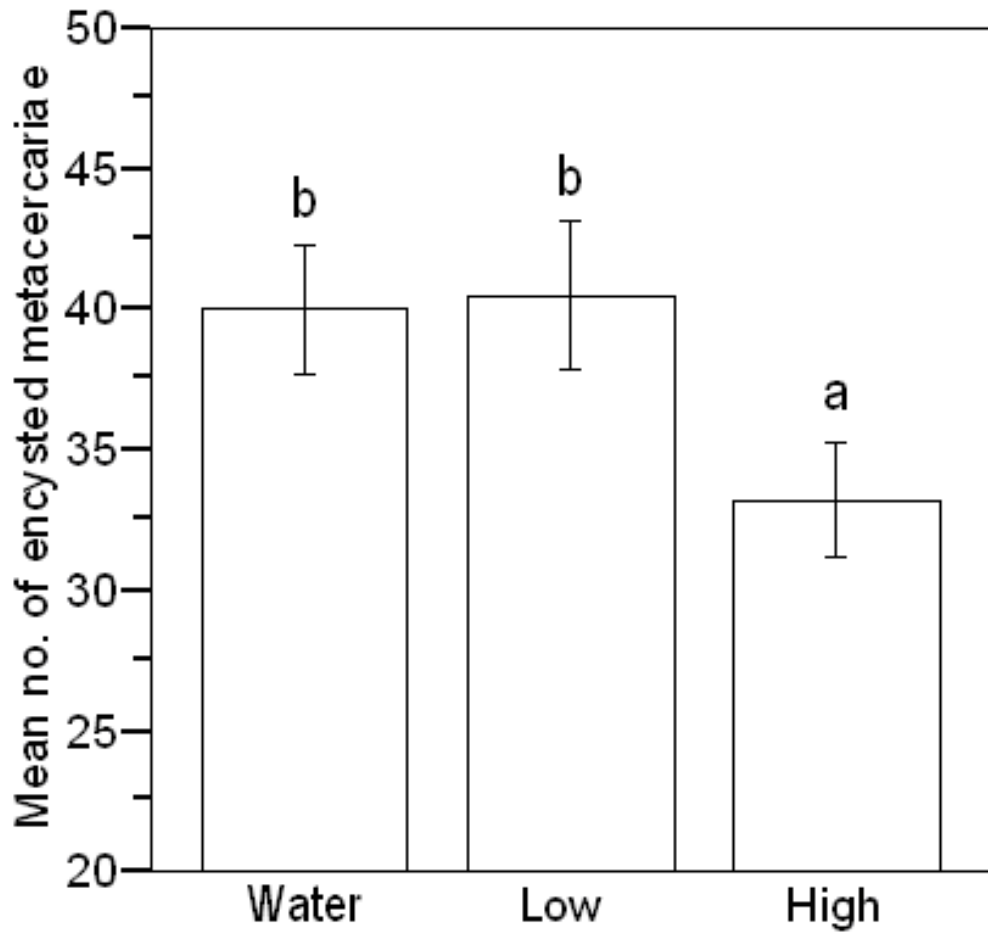
Figure 4.2. Mean (± SE) number of encysted metacercariae in minnows exposed to 1) cercariae immersed in dechlorinated tap water (control), 2) cercariae immersed in a low concentration of minnow skin extract (0.1 cm^2 of skin/ 10-mL of water) and 3) cercariae immersed in a high concentration of minnow skin extract (1 cm^2 of skin/10-mL of water) over two time periods (0-min and 30-min). Shared letters above bars indicate no difference (p < 0.05) in Tukey post-hoc pairwise comparisons.
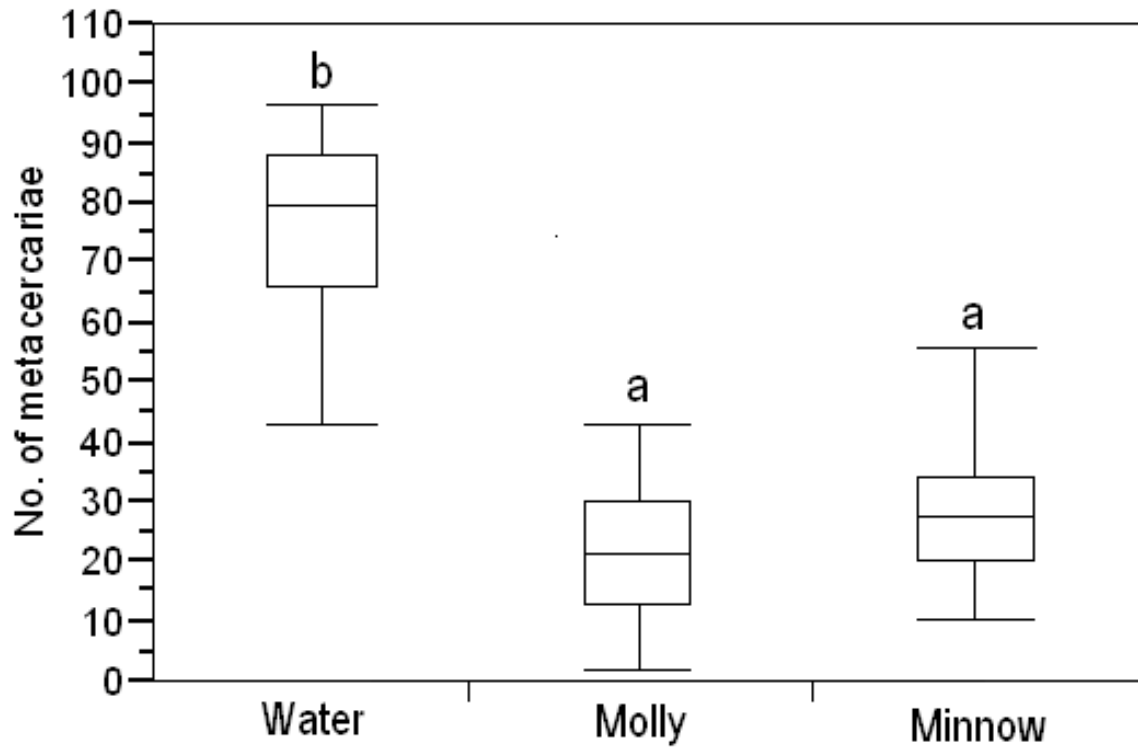
Figure 4.3. Median (± Quartiles and Range) number of encysted metacercariae in minnows exposed to 1) cercariae immersed in non-ostariophysan skin extract (mollies, 1 cm^2 of skin/10-mL of water), 2) cercariae immersed in ostariophysan skin extract (minnow, 1 cm^2 of skin/10-mL of water) and 3) cercariae immersed in dechlorinated tap water (control). Shared letters above bars indicate no difference (p < 0.05) following Kruskal-Wallis multiple comparisons (Siegel & Castellan, 1998).
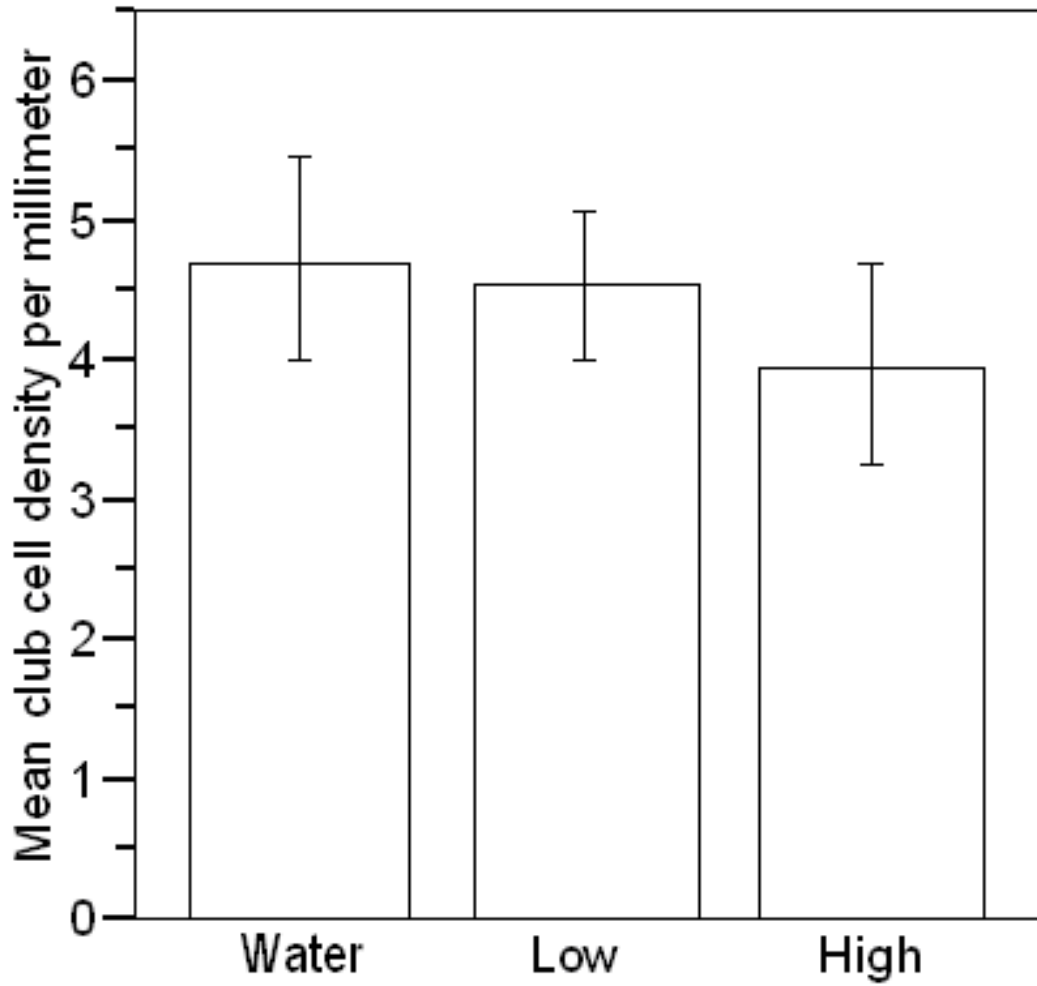
Figure 4.4. Mean (± SE) number of club cells per mm in nape of minnows exposed to 1) 0 cercariae, 2) 30 cercariae and 3) 150 cercariae.
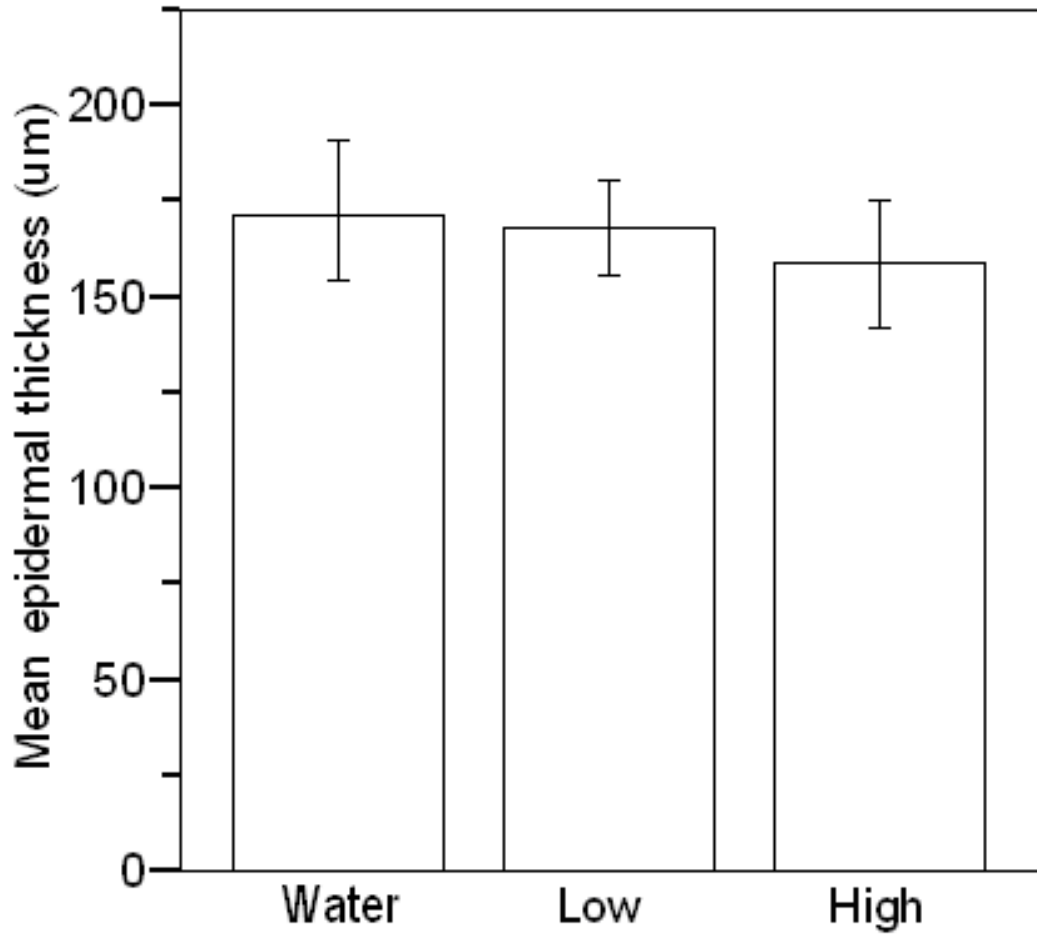
Figure 4.5. Mean (± SE) epidermal thickness (μm) of nape in minnows exposed to 1) 0 cercariae, 2) 30 cercariae and 3) 150 cercariae.

Chapter 5. General Conclusions

Natural selection should favour hosts that can avoid costly parasites. This is a simple and intuitive prediction, yet it is very rarely tested within natural host/parasite interactions, especially those involving aquatic hosts. The combined set of experiments in my thesis represent one of the first tests of this general prediction in a fish/trematode system (Chapters 2 and 3) and they represent one of the first tests of a key mechanism potentially leading to behavioural responses (Chapter 4). More specifically, I tested three hypotheses that seek a linkage between a fish's exposure to parasite larvae and potential behavioural responses. In Chapter 2, I tested the hypothesis that shoaling reduces an individual minnows' risk of cercarial exposure. In Chapter 3, I used a standard learning paradigm to test the hypothesis that minnows associate prior exposure to cercariae with avoidance upon re-exposure. The experiments in Chapter 4 provide a very rare test of the 'antipathogen hypothesis' (Chivers *et al.*, 2007) for a role of epidermal club cells in the detection and protection of cercariae by minnows.

Results from the series of experiments completed in this thesis provide mixed support for these hypotheses. In general, there was support for the first hypothesis. Thus, minnows exhibit specific avoidance behaviours when exposed to both live and dead cercariae. Minnows exposed to cercariae in aquaria reduced their overall activity following subsequent exposure by approximately 50% compared to water controls (Chapter 3). This result is important because it is the first demonstration that fish can detect cercariae cues and reduce their activity accordingly. Previous studies of fish

behavioural responses to trematode cercariae are unable to distinguish whether fish hosts can detect cercariae prior to infection or as a consequence of infection (see Karvonen *et al*., 2004 for example).   My study provides empirical evidence that fish can detect cercariae, via visual or olfactory cues, prior to infection and thus can respond with avoidance behaviours.

An equally important result from Chapter 3 is the preliminary evidence for a learned response to cercariae. This is an important advance because it is the first test of the classical learning paradigm for trematode/fish interactions. Minnows can be conditioned to respond to novel cues with avoidance behaviours by first associating the stimuli of a novel cue with chemical cues that indicate risk (see review by Brown, 2003). This form of conditioning, or learning, occurs in fathead minnows when neutral stimuli such as sound, light, or chemical cues of non-predatory fish, or predatory fish become associated with predation risk (Chivers & Smith, 1994; Yunker *et al*., 1999; Wisenden *et al*., 2007). My research extends this phenomenon to include host-parasite interactions.

This result opens up a new area of study in fish/parasite ecology. Follow up studies might focus on the specificity of this response. That is, can minnows associate exposure to *Ornithodiplostomum* sp. cercariae with subsequent exposure to other species of cercariae that infect minnows? Can they discriminate species of cercariae that infect minnows, from the many species of cercariae that they must encounter in natural habitats that do not? Can minnows associate environmental signals of infection (presence of infected snails, water temperature, substrate characteristics etc.) with risk and then

engage in appropriate avoidance behaviours? Following my results that indicate reduced host activity following re-exposure to cercariae cues, these and many other questions can be posed using similar methods.

My tests of the shoaling hypothesis received mixed support. Laboratory populations of minnows formed tighter shoals when exposed to trematode cercariae (Chapter 2). Although shoaling as an antiparasite behaviour has been previously documented for fish exposed to ectoparasites (Poulin & FitzGerald, 1989), my research is the first to record this specific behaviour in response to trematode cercariae. This is an important result, first because it confirms that minnows can detect cercariae in the water column and respond behaviourally, and second because it provides preliminary evidence that shoaling behaviour has the potential to reduce rates of infection. However, the design of my experiment could not distinguish whether minnows are responding to the risk of infection by cercariae, or are simply responding to a foreign 'substance' in the water column that elicits a general stress response. Future studies will need to expand on this result by incorporating the approach used in Chapter 3. Thus, do minnows shoal in response to cercariae-conditioned water? Does variation in the magnitude of the shoaling response vary with species of cercariae, with the numbers of cercariae, or their size? Is the magnitude of the shoaling response conditional upon prior exposure, following the results of Chapter 3?

Results from my tests of the antiparasite role of shoaling were tantalizing, but inconclusive (Chapter 3). Although the overall distribution of metacercariae counts in

fish confined to artificial shoals tended to be lower than those constrained to individual container (Chapter 2, Figs. 2.4 and 2.5), differences in mean intensity between the two groups were not significant. However, given the enormous variation in counts between and within individual mesocosms (and between individual fish), the power to detect a significant effect was low. Perhaps a key advance of this experiment lies in the demonstration of the potential value of manipulative tests of the shoaling hypothesis under semi-natural conditions. Each individual survived within the constrained cages and each was exposed to large numbers of cercariae. Slight modifications to design that might decrease the exposure rate (e.g., addition of fewer infected snails) would help to restrict the magnitude of variation in metacercariae counts between individual ponds. Results of this experiment indicate that the placement of artificial cages within natural wetlands would be feasible. Thus, the shoaling hypothesis could conceivably also be tested under natural conditions of cercarial transmission.

My tests of the antipathogen hypothesis (Chivers et al., 2007) for a role for club cells were not supportive. However, an important advance demonstrated in chapter 4 is that some component of fish skin reduces cercariae infectivity by 61-68%. This result, especially its magnitude, is striking. A large number of factors has been demonstrated to reduce cercariae infectivity, but almost all are associated with extrinsic factors such as temperature, wave action, ultraviolet radiation, and water quality. My results show that a component of fish epidermis that is shared by both ostariophysan hosts (that contain epidermal club cells) and non-ostariophysan non-hosts have a very large impact on cercariae infectivity. One possibility is that antipathogenic agents associated with mucus

cells reduce infectivity in a manner similar to the way they inhibit infectivity of monogenean trematodes and other aquatic pathogens (reviewed by Shephard, 1994, see also Chapter 4). This was an unexpected result of my thesis. It is clear that more detailed studies at the epidermis/cercariae interface need to be considered to identify the component(s) of the epidermis that are involved in reducing infectivity.

Although some component of the epidermis reduces cercariae infectivity, it is unlikely that club cells (possessed by ostariophysans) are responsible. Skin extract removed from non-ostariophysans, that do not contain club cells, reduced infectivity to the same extent as skin extract removed from minnows. Further, there was no association between club cell density and metacercariae intensity. Both results contradict the results of Chivers *et al*. (2007) on a similar fish/cercariae system. Given that there have only been two empirical tests of this hypothesis, it is too early to make definitive conclusions. However, my experiment differed from the experiment completed by Chivers *et al*. (2007) in that I exposed minnows to a naturally-occurring, common, and host-specific parasite. Therefore, I conclude that based upon current evidence, epidermal club cells do not play a protective role against common cercariae.

One fundamental assumption underlying this thesis is that reduced host activity lowers a host's exposure to trematode cercariae. As seen in Chapter 3, minnows greatly reduce their activity upon re-exposure of trematode cercariae compared to water controls. This result suggests reducing activity is a behaviour preferred by minnows to reduce their chance of exposure. Unfortunately however, I did not specifically test for this causal

relationship. Evidence from previous studies on similar host/parasite interactions provides the empirical basis for this relationship (Poulin *et al.*, 1991; Thiemann & Wassersug, 2000). Similarly, my anecdotal observations strongly support the notion that active minnows are more at risk of infection than stationary ones. In the pond experiment, one minnow escaped from its cage. This minnow harboured 425 metacercariae while the average intensity of infection in the other 9 minnows was 146. This result strongly suggests that reduced minnow activity is an effective strategy to restrict exposure to *Ornithodiplostomum* sp. Thus, minnows that move more come into contact with more cercariae. Because shoaling is often correlated with a decrease in activity (Mathis & Smith, 1993), future experiments might look at whether reduced activity (Chapter 3) and shoaling (Chapter 2) in combination, might act in concert to reduce a minnows' risk of infection. The overlap between both shoaling and reduced activity may combine the advantages of both behaviours and thus provide a more potent form of antiparasite protection.

Despite my empirical evidence showing that minnows alter their behaviour when exposed to trematode cercariae, I know little of the degree to which these behaviours are important for minnows in the wild. In laboratory conditions, the concentrations of cercariae that I used are likely much higher than natural. For minnows in areas of lower concentrations of cercariae, such as in the field, the costs of participating in avoidance behaviours might exceed the costs of infection and thus minnows may not engage in avoidance behaviours to the same degree as those observed in the laboratory. Moreover, in the field, minnows encounter a variety of cues from confounding sources (i.e., predators, other parasites, and food sources) that may mask the cues of cercariae or at the

least make it difficult to single out and locate cercariae. Likewise, minnows housed in laboratory conditions experience considerably higher stress levels than their wild counterparts and as a result may partake in avoidance behaviours more frequently. Due to discrepancy between laboratory and field settings, I cannot be certain that avoidance behaviours observed are common in the field. However, my study provides some of the first empirical evidence that minnows at least possess the capacity to elicit avoidance behaviours when exposed to trematode cercariae.

Although minnows participated in avoidance behaviours throughout my experiments, I do not understand the evolutionary origin of these behaviours. Are minnows responding to infection specifically or are the behaviours observed artifacts of behaviours modified for predator avoidance? Both shoaling and reduced activity are also effective antipredation behaviours (Mathis & Smith 1993; Wisenden et al. 1999). Can minnows differentiate between predators and pathogens, and if so, can they moderate the degree of their behavioural response? Predators have a more immediate impact on host fitness compared to parasites and thus may merit a stronger behavioural avoidance response. The degree to which minnows respond to stress may be directly related to the risk to their fitness. Comparative studies that assess the link between predator and pathogen avoidance are in short supply (see Thiemann & Wassersug, 2000, for exception) but should be considered more carefully in future studies to develop a better understanding of the ecology of animals that serve as both prey to predators and hosts to parasites.

For minnows, the avoidance of both predators and parasites may share similar negative consequences. For example, predator-induced reductions in fish activity have strong negative effects on the foraging behaviour of individuals. Similar costs are likely to exist for antiparasite behaviours. Thus, avoidance behaviours that alter host activity (Chapter 3, Poulin & FitzGerald, 1989; Theimann & Wassersug, 2000; Karvonen *et al*., 2004) should also be expected to reduce foraging opportunities. Likewise, in aquatic systems, antiparasite behaviours may conflict with antipredator behaviours. Participating in either parasite or predator avoidance often comes at the cost of increased exposure to the other (Baker & Smith, 1997; Thiemann & Wassersug, 2000; Mikheev & Pasternak, 2006). However, there are very few tests of potential trade-offs between parasite and predator avoidance strategies and the *Pimephales promelas/Ornithodiplostomum* sp. interaction is no exception. Lastly, it is also conceivable that participating in avoidance behaviours in response to one parasite may come at a cost of increased exposure to others. For example, inactivity induced by motile cercariae may lead to increased exposure to parasites that require direct contact with substrate. Thus, selection may not exist for specific antiparasite behaviours directed to one species, but for a low-level, generalized response to parasite risk.

In conclusion, this work opens new avenues of research into multidimensional decision making in behavioural ecology. Previous literature in behavioural ecology has primarily focussed on simplistic two-dimensional trade-offs, i.e. predation risk/foraging trade-offs (Sih, 1982), or predation risk/reproduction trade-offs (Magnhagen, 1995) or shoaling/parasite risk trade-offs (Krause, 2002). My research adds to the seminal work of

Thiemann & Wassersug (2000) in adding a higher level of complexity, and thus greater ecological realism to the understanding of the behavioural ecology of aquatic organisms. Achieving this level of complexity requires capitalizing on a host/parasite interaction that is amenable to experimentation, such as the *Pimephales promelas*/*Ornithodiplostomum* sp. system. Parasite risk, predation risk and conspecific social interactions operate simultaneously that all combine to exert dynamic selection forces on the behavioural ecology of aquatic organisms. Using trematode cercariae, I have determined minnows participate, and invest in, highly complicated defensive strategies. However, more work is needed to determine the extent to which these antiparasite defenses ameliorate the costs of infection and how natural selection operates among these competing demands on minnow ecology.

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APPENDIX

Summary statistics of one-way ANOVA comparing the difference between shoal cohesion of minnows exposed to 400 cercariae and minnows exposed to water (Chapter 2)

Source	DF	SS	MS	F Ratio	p-value
Treatment	1	10.267	10.267	9.198	0.005
Error	28	31.254	1.116		
C. Total	29	41.521			

Level	Number	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Cercariae	15	-1.37	1.18	0.304	-2.022	-0.718
Water	15	-0.20	0.92	0.238	-0.710	0.310

Summary statistics of paired t-test comparing the difference between infection levels of minnows constrained in shoal and non-shoal configurations (Chapter 2)

Non-Shoal Mean	119.15
Shoal Mean	103.34
Mean Difference	15.81
Std Err	16.240
Upper95%	54.214
Lower95%	-22.589
N	8
Correlation	0.675
t-Ratio	0.974
DF	7
p-value	0.363

Summary statistics of one-way ANOVA comparing the difference in activity levels between naïve, experienced, and water control minnows (Chapter 3)

Source	DF	SS	MS	F Ratio	p-value
Treatment	2	13205.333	6602.670	5.271	0.014
Error	21	26304.500	1252.600		
C. Total	23	39509.833			

Level	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Experienced	8	-27.25	43.34	15.322	-63.480	8.982
Naïve	8	16.75	31.25	11.050	-9.380	42.879
Water	8	26.75	30.05	10.623	1.630	51.869

Summary statistics of one-way ANOVA comparing the difference in vertical distribution between naïve, experienced, and water control minnows (Chapter 3)

Source	DF	SS	MS	F Ratio	p-value
Treatment	2	0.736	0.368	0.919	0.414
Error	21	8.415	0.401		
C. Total	23	9.151			

Level	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Experienced	8	-0.20	0.78	0.277	-0.857	0.452
Naïve	8	0.23	0.72	0.253	-0.372	0.824
Water	8	0.03	0.28	0.098	-0.202	0.262

Summary statistics and effect tests of two-way ANCOVA comparing metacercarial intensities between minnows exposed to cercariae submersed in a 2 concentrations of minnows skin extract (low, high) and water over two time periods (0-min, 30-min) (Chapter 4)

Source	DF	SS	MS	F Ratio	p-value
Model	6	2908.526	484.754	3.622	0.003
Error	73	9768.962	133.821		
C. Total	79	12677.487			

Source	Nparm	DF	SS	F Ratio	p-value
Dose (Water, Low, High)	2	2	1320.712	4.935	0.010
Time (0-min, 30-min)	1	1	377.677	2.822	0.097
Total Length	1	1	1451.665	10.848	0.002
Time*Dose	2	2	247.309	0.924	0.402

Level	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Water	29	25.97	2.96	0.549	24.840	27.091
High	23	28.09	4.20	0.875	26.271	29.903
Low	28	26.75	2.49	0.470	25.785	27.715

Summary statistics of Kruskal-Wallis one-way ANOVA comparing metacercarial intensities between minnows exposed to cercariae submersed in ostariophysan, non-ostariophysan skin extract, and water (Chapter 4)

KW	DF	p-value
60.9341	2	<.0001

Level	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Non-ostariophysan	29	21.72	10.93	2.030	17.566	25.882
Ostariophysan	32	28.94	12.36	2.185	24.481	33.394
Water	30	77.20	13.69	2.500	72.087	82.313

Summary statistics of one-way ANOVA comparing club cell densities in minnows exposed to 2 levels of infection (low and high) and water (Chapter 4)

Source	DF	SS	MS	F Ratio	p-value
Treatment	2	6.311	3.156	0.369	0.693
Error	57	487.012	8.544		
C. Total	59	493.323			

Treatment	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Water	20	4.72	3.16	0.706	3.238	6.194
High	20	3.96	3.17	0.710	2.475	5.446
Low	20	4.55	2.36	0.528	3.445	5.657

Summary statistics of one-way ANOVA comparing epidermal thickness (μm) in minnows exposed to 2 levels of infection (low and high) and water (Chapter 4)

Source	DF	SS	MS	F Ratio	p-value
Treatment	2	1852.650	926.330	0.189	0.828
Error	57	279054.560	4895.690		
C. Total	59	280907.210			

Treatment	N	Mean	Std Dev	Std Err	Lower 95%	Upper 95%
Water	20	172.72	77.54	17.339	136.430	209.010
High	20	159.37	74.61	16.683	124.450	194.280
Low	20	168.33	55.75	12.465	142.240	194.420